Aus der Klinik für Dermatologie, Allergologie und Venerologie
der Universität zu Lübeck
Direktor: Prof. Dr. med. D. Zillikens
und dem Lübecker Institut für Experimentelle Dermatologie
Wissenschaftlicher Leiter: Prof. Dr. med. Dr. rer. nat. Enno Schmidt

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Development and testing of a novel *ex vivo* assay for studying "pathological" wound healing in human skin

Inauguraldissertation
zur
Erlangung der Doktorwürde
der Universität zu Lübeck
- Aus der Sektion Medizin –

Vorgelegt von Hannah Post
aus Gronau, Deutschland

1

Lübeck
2018

1. Berichterstatterin: Prof. Dr. med. vet. Jennifer E. Hundt
2. Berichterstatter/ -in:

Tag der mündlichen Prüfung

Zum Druck genehmigt Lübeck, den

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Abbreviations

3D	=	three-dimensional
AIDS	=	acquired immune deficiency syndrome
AGE/s	=	advanced glycation end-product/s
ATP	=	adenosine triphosphate
AWMF	=	*Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften*
bFGF	=	basic fibroblast growth factor
BL	=	basal lamina
CK	=	cytokeratin
$CoCl_2$	=	cobalt(II)-chloride
DAPI	=	4’6-diamidin-2-phenylindol
ET/s	=	epithelial tongue/s
EPO	=	erythropoetin
ECM	=	extracellular matrix
EGF	=	epidermal growth factor
ESC/s	=	epidermal stem cell/s
FGF	=	fibroblast growth factor
FGFR1	=	fibroblast growth factor receptor 1
FITC	=	Fluorescein Isothiocyanate
GF/s	=	growth factor/s
GLUT	=	glucose transporter
H_2O_2	=	hydrogen peroxide
H&E	=	haematoxylin and eosin
HIF-1α	=	hypoxia inducible factor-1 alpha
HF/s	=	hair follicle/s
HO^-	=	hydroxyl ion
I^-	=	iodide
I_2	=	iodine
IF	=	immunofluorescence
IFE	=	interfollicular epidermis
IFNγ	=	interferon gamma
IGF	=	insulin like growth factor

IL	=	interleukin
IR	=	immunoreactivity
KC/s	=	keratinocyte/s
KGF	=	keratinocyte growth factor
LDH	=	lactat dehydrogenase
LN5	=	laminin 5
mRNA	=	messenger ribonucleic acid
MMP/s	=	matrix metalloproteinase/s
NIS	=	sodium-iodide symporter
NO	=	nitric oxide
O^-_2	=	superoxide anion
PBS	=	phosphate buffered saline
PM	=	“pathological” medium
PM+T4	=	“pathological” medium + thyroxine
PAR	=	protease-activated receptor
PAS	=	periodic acid-schiff
PDGF	=	platelet-derived growth factor
ROS	=	reactive oxygen species
SM	=	standard medium
SM+T4	=	standard medium + thyroxine
T3	=	triiodothyronine
T4	=	thyroxine
TBG	=	thyroid-binding globulin
TBS	=	tris buffered saline
TdT	=	terminal desoxynucleotidyl transferase
TGF	=	transforming growth factor
TH/s	=	thyroid hormone/s
TH-R	=	thyroid hormone receptor
TNF-α	=	tumor necrosis factor-alpha
TPO	=	thyroid peroxidase
TRE	=	thyroid hormone response element
TRH	=	thyrotropin releasing hormone
TRH-R/s	=	thyrotropin releasing hormone receptor/s

TSH	=	thyroid-stimulating hormone
TSH-R	=	thyroid-stimulating hormone receptor
TTR	=	transthyretin
TUNEL	=	Tdt-mediated dUTP-biotin nick end labeling
UV	=	ultraviolet
VEGF	=	vascular endothelial growth factor
vs.	=	versus
WH	=	wound healing

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3 Introduction

3.1 Structure and function of human skin

The skin is a huge and multifunctional organ and a variety of skin properties are in the focus of current research. Besides its barrier function and protection against external threats, such as ultraviolet (UV) radiation, thermal, mechanical and chemical forces the local immune system, a highly complex structure and composition of cell systems defends the body from pathogen agents (Krieg and Aumailley, 2011; Madison, 2003; Proksch et al., 2008; Yazdi et al., 2016). The skin also controls fluid balance and fulfils endocrine functions regulating the metabolism of hormones such as vitamin D, thyroid hormones (THs) and a high number of neurohormones (Chuong et al., 2002; Langan et al., 2013; Nejati et al., 2013; Ramot and Paus, 2014; Xue et al., 2015). Moreover, the skin functions as an organ of perception providing the sensations of pain, touch, vibration, temperature and itch and also plays a crucial role for social and sexual communication (McGlone and Reilly, 2010).

With average dimensions of 2 m^2 and 8-10 kg it is not only the biggest organ but also the largest gland of a human being (Zouboulis and Bornstein, 2013). The arrangement of the three skin layers: epidermis, dermis and subcutis conforms to their structure and function. Hair, nails and glands are skin appendages and play a pivotal role for skin integrity. **Figure 1** shows a cross-section of all skin layers and the associated skin appendages. The epidermis defines the protective shield by its multi-layered squamous epithelium. Structural support and nutrition of the epidermis are key functions of the well-perfused and highly innervated dermis. The adipose layer of the skin is the subcutis which is also well-perfused and innervated (Agache et al., 2017; Sterry et al., 2006).

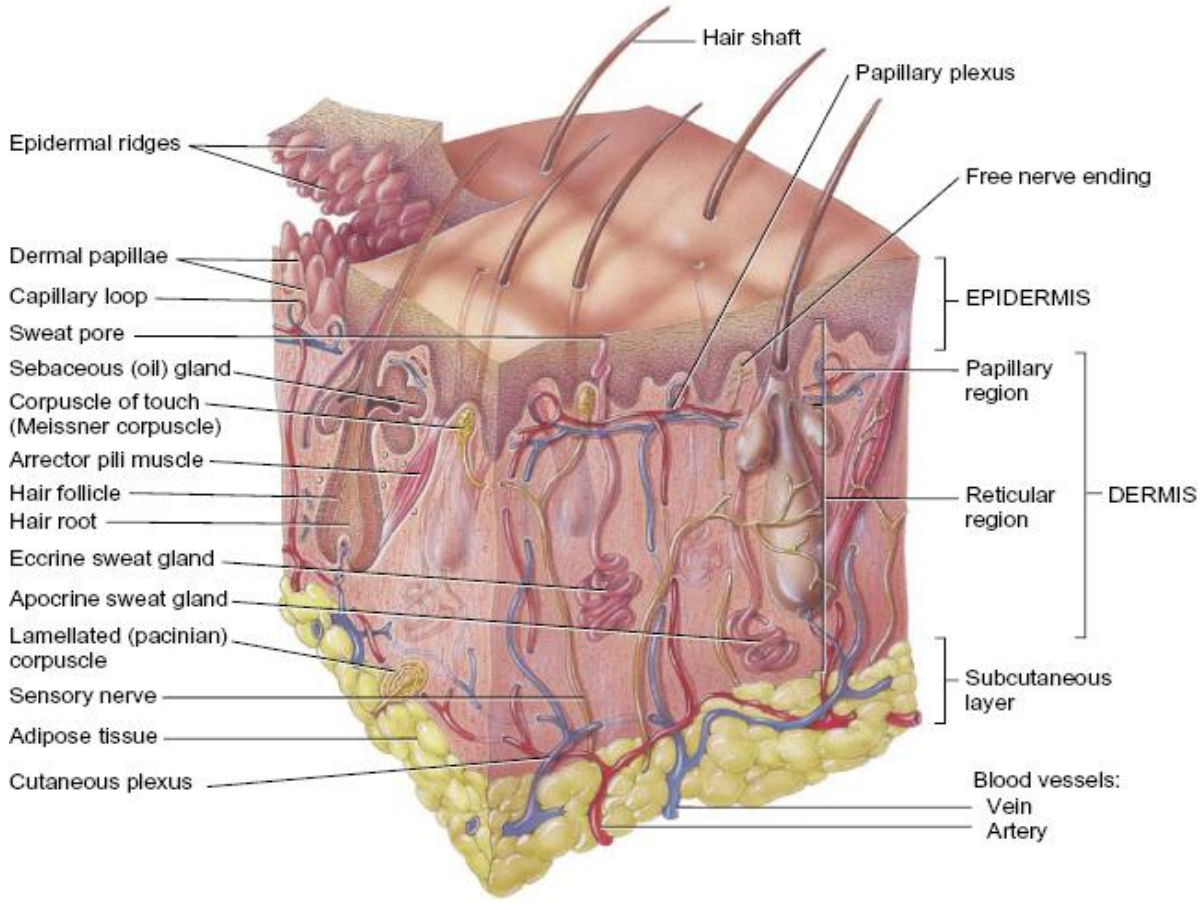

Figure 1 Cross-sectional view on the structure of human skin

The figure shows a three-dimensional section of human skin and provides an overview of the arrangement of the three skin layers (epidermis, dermis and subcutis). It is a simplified draft of the complex network between skin cells, appendages, blood vessels and touch receptors. It becomes clear that the dermis plays a dominant role in the size and amount of processes taking place inside the skin. Taken from: (Tortora and Tortora, 2014), “Principles of Anatomy and Physiology”, 14th edition, Chapter 5.

3.1.1 Life-cycle of a keratinocyte within the epidermis

The dominating cell types of the epidermis are keratinocytes (KCs) (95%). They are tightly connected to each other by cell adhesion molecules called desmosomes which are anchored to the actin filament of the KCs to fulfil the barrier function of the epidermis (Proksch et al., 2008). Other cell populations are immune cells like T cells and Langerhans cells, highly sensitive Merkel nerve ending functioning as mechanoreceptors and the pigment-producing melanocytes in the *stratum basale* which regulate melanin synthesis and transfer in purpose to protect the nuclei of KC from UV radiation (Plonka et al., 2009; Slominski et al., 1993). The epidermis is

abundantly innervated whereas blood- and lymphatic vessels do not exist in this layer (Arda et al., 2014; Sterry et al., 2006). The epidermis is structured by four–five layers starting with the *stratum basale,* the basal lamina (BL) at the bottom. The composition of epidermal cells and layers is shown in **Figure 2**. The BL is built up out of undifferentiated proliferating KCs and a highly complex extracellular matrix (ECM) network consisting of type 4 collagen, laminin (LN) and other structural proteins (Breitkreutz et al., 2013). The exchange of bioactive substances, like growth factors (GFs) or cytokines between dermis and epidermis is controlled by the BL. Regenerative processes are also determined by stem and progenitor cells which are located in the BL (Boehnke et al., 2012). To maintain epithelial polarity and morphogenesis, KCs start their differentiation upwards from the BL to the *stratum spinosum* (prickle cell layer) where KCs are tightly connected by desmosomes. This is where the keratinisation process starts and the cell-division of the skin cells ends (Herrmann and Trinkkeller, 2015). The subsequent *stratum granulosum* (granular cell layer) is characterised by flattened KCs that are enriched with stabilising proteins and keratohyalin granules, which build a water-resistant barrier but also prevents the underlying structures from dehydration (Welsch and Deller, 2010). The *stratum lucidum* is particularly expressed in plantar and palmar skin. Nuclei and other cell organelles pass through a complex and fast apoptosis mechanism which makes it appear bright in a non-stained sample under the microscope. Finally, the *stratum corneum* covers the epidermis with the aged KCs that have rejected their nuclei and cell organelles and functions as a “protective-shield”. To guarantee an effective barrier against chemical and physical environmental influences, avital KCs are still connected by desmosomes and contain keratin filaments. Intercellular spaces are filled with a protein matrix and lipids (Wato et al., 2012). It takes 28-30 days for a complete renewal of the epidermis (Gantwerker and Hom, 2011).

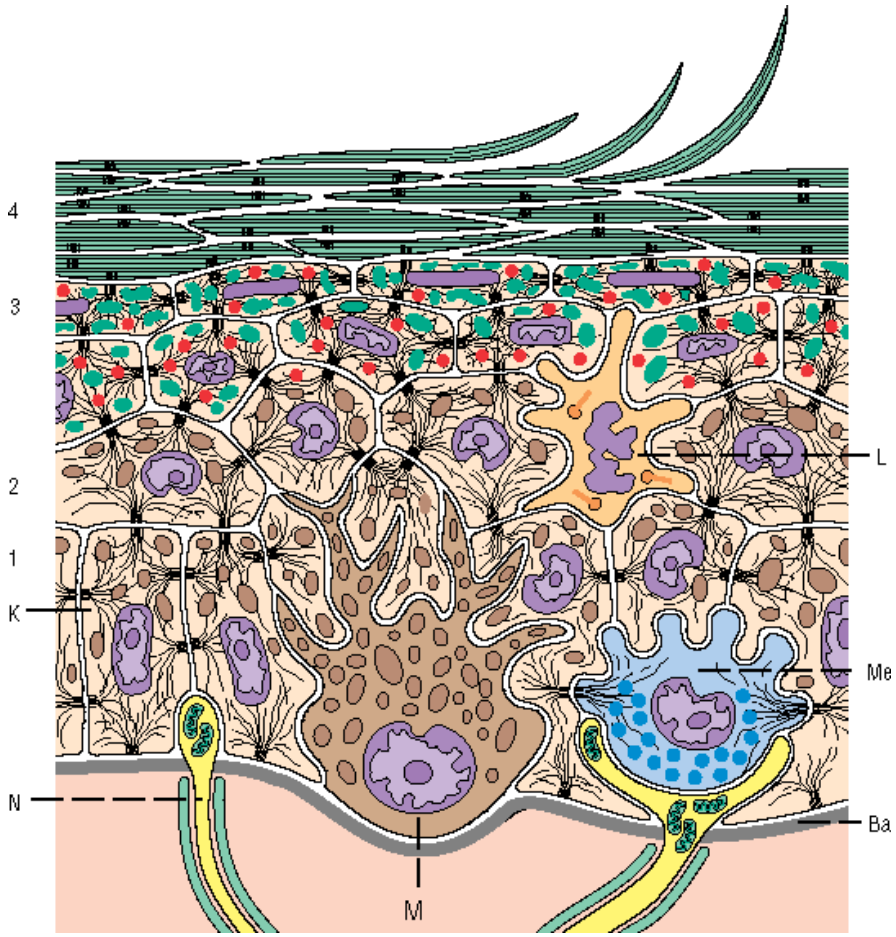

Figure 2 Cells of the epidermis

The figure shows an outline of a longitudinal section of the epidermis. The epidermal layers are numbered from 1-4 (1: s*tratum basale*, 2: s*tratum spinosum*, 3: s*tratum granulosum*, 4: *stratum corneum,* s*tratum lucidum* is not shown here). Keratinocytes (K) make up the largest amount of epidermal cells. Melanocytes (M) located in the s*tratum basale* produce melanosomes which are also transferred to the K. Merkel cells (Me) also represented in the *stratum basale* are mechanoreceptors responsible for the sensation of touch and are connected to somatosensory afferent nerve fibres (N). Langerhans cells are mostly found in the *stratum spinosum* and form a dendritic net for the detection of antigens.

Taken from: (Welsch and Deller, 2010), Lehrbuch Histologie, 2nd edition, Chapter 16.

3.1.2 Maintenance and structural role of the subepidermal tissue

Underneath the epidermis, the dermis and the subcutis are responsible for structuring the skin. Dermal collagen-fibres provide textile strength and the vascular system of blood and lymphatic vessels serves for the nutrition of the non-perfused epidermis. The subcutaneous fat which is divided into lobuli by fibrinous septa functions as an energy reserve for the skin. The dermis consists of two layers. The *stratum papillare* is connected to the epidermis and the *stratum reticulare* is connected to the subcutis.

Connective tissue papilla (papillary dermis) are intertwined with epidermal “rete ridges” for a resilient mechanical connection and to guarantee the nutrition of the epidermis. The *stratum papillare* is enriched by blood and lymphatic vessels. The lymphatic system of the skin starts here and drains the large interstitium which is located in intercellular spaces and contains a gel-like liquid. It is also where the majority of sensory nerve fibres of the skin are located. Collagen type III is the dominating connective tissue fibre in this area. Major cell types are mast cells, macrophages, other dendritic cells and fibroblasts (Welsch and Deller, 2010). During wound healing (WH) fibroblasts play a major role in the production of ECM and collagen fibres and in the conversion to contractile cells (myofibroblasts) (Vedrenne et al., 2012). In the second dermal layer the *stratum reticulare* the predominating structural protein fibres are collagen type I and IV, which are connected to elastin fibres and also determine, depending on the water content, the firmness of the skin (Gaboriau and Murakami, 2001). Skin appendages like sebaceous and sweat glands, the bulge of a hair follicle (HF) and mechanoreceptors that enable us to perceive vibration, touch and temperature, are also embedded in the dermis. Underneath the dermis, the subcutis is composed of adipose tissue, which is separated by loose connective tissue septa that contain blood and lymphatic vessels and nerve fibres. The structure of the whole adipose and connective tissue body varies in the different areas of the body. For example, in plantar and palmar areas it functions as structural fat whereas abdominal fat serves as depot fat. Thermoregulation, energy storage and the regulation of food uptake via leptin is navigated by the adipose tissue (Cerman et al., 2008; Klein et al., 2007). The hormone leptin also becomes an important point of interest in the context of WH when considering research indicating that its release may promote WH (Negrão et al., 2012). Furthermore, adipose tissue-derived stem cells have been analysed to offer promising advantages in regenerative medicine (Beeson et al., 2011).

3.1.3 Role of skin appendages in human skin

Skin appendages - hair, nails and glands - rise from the BL and grow into lower parts of the dermis and subcutis. In case of nails and hairs the keratinised part protrudes over the skin level. Regarding hair structure, the part above the skin level is called hair shaft and the part inside the skin is defined as the actual HF. The distal part is called infundibulum where apocrine and sebaceous glands are attached to the hair canal

(Schneider et al., 2009). In the isthmus region, arrector pili muscle inserts and at the same level the bulge region is located, containing epithelial stem cells (ESCs) (Kloepper et al., 2008). The proximally located hair bulb is composed of the inner and outer root sheath and surrounded by the connective tissue sheath including small blood vessels and nerves. The most proximal part of the HF is the dermal papilla which is composed of specialised mesenchymal cells. Highly proliferative fibroblasts interact with melanocytes and KCs and contribute to produce and maintain the hair shaft (Langan et al., 2015; Paus and Cotsarelis, 1999). Hair pigmentation is managed by melanosomes which the KC of the HF receive from specialised HF melanocytes (Slominski et al., 2005). The composition of a HF is demonstrated in **Figure 3**. HFs undergo a frequent cycling during the lifetime of an organism. This is determined by three stages, anagen (active growth phase), catagen (regression phase) with a duration that depends on the body region and telogen (resting phase) (Paus, 2010). Referring to cutaneous WH phases, there seems to be a close relation to HF phases. Anagen HFs appear to have a promoting effect on WH (Ansell et al., 2011; Takeo et al., 2015). Further, physiological evolutional functions of HFs are: protection against UV radiation, heat, temperature and humidity regulation.

Human skin contains different types of glands which are located in determined body regions. Eccrine sweat glands are tubular, unbranched glands with a tight lumen and can be located most downwards in the subcutis. The eccrine sweat glands are responsible for the body temperature regulation by thermal sweating and abundant in all cutaneous body parts. Emotional sweating mainly takes place at the forehead, axillary, palmar and plantar where apocrine sweat glands are located (Welsch and Deller, 2010). The holocrine sebaceous glands are associated to the HF by their wide lumen. They are also widespread all over the integument besides palmar and plantar and produce a lipid layer to reduce water permeability (Niemann and Horsley, 2012; Zouboulis, 2003). The cutaneous glands are also assumed to have regulating functions for WH processes. It was proven that they imply nestin-positive stem cells which promote WH in murine skin (Danner et al., 2012) and in wounded organ cultured human skin (med. diss., Zhang, 2013). Eccrine sweat glands and pilosebaceous units also have been reported to contribute to reepithelialisation of wounded human skin *in vivo* (Rittié et al., 2013).

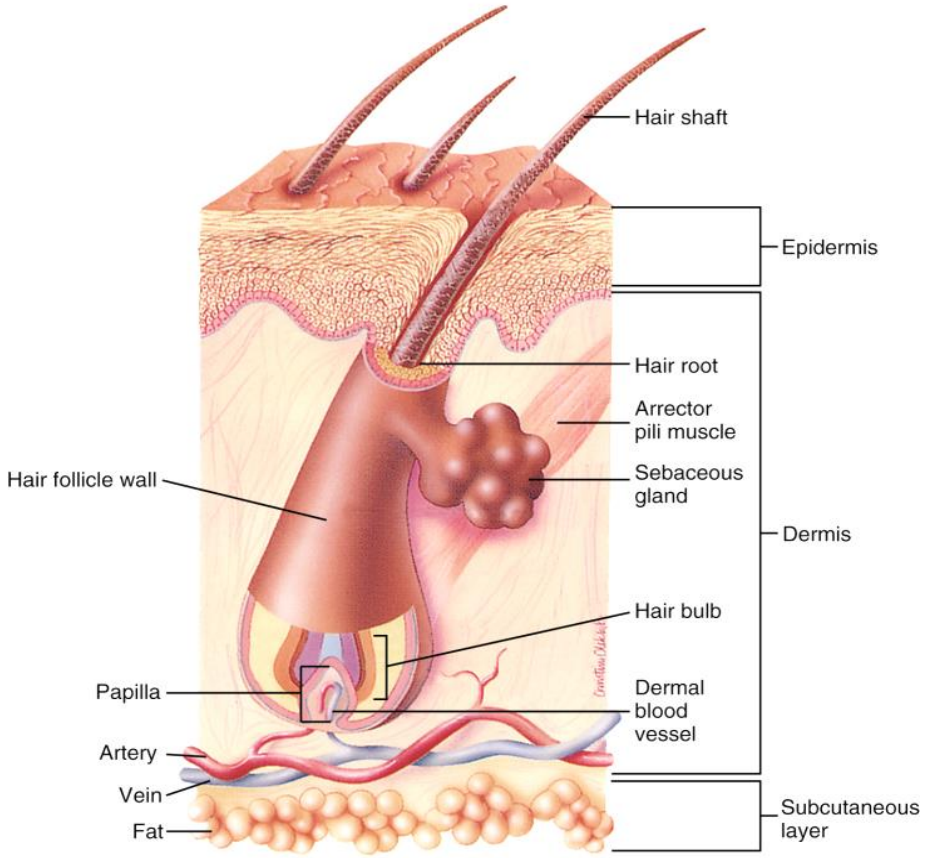

Figure 3 Insertion of the human hair follicles inside the skin

The hair follicle (HF) is the “manufacturing site” of the hair. This transverse section shows how the HF is embedded in the tissue and also linked to other skin appendages or blood vessels. In the bulge region, an insight on the detailed arrangement of HF layers is provided. Taken from: http://koreesa.co.uk/hair-follicle, day of access: 02.04.2018.

Nails are inserted into the cutis at the endphalanx of toes and fingers built up of a differentiated corneal layer. Contrary to HFs nails grow in a constant manner. The nail plate sprouts from proliferating cells of the nail matrix and it is proximal and lateral framed by a skin bulge (Welsch and Deller, 2010). In the nail matrx, horizontal capillaries are located and microscopic changes can be analysed for specifying diseases like collagenoses (Haneke, 2014) and melanocytes are located in this region. The function of nails can be summarised as a protection of the phalanx, itching tools and abutments for the fingertips for the sense of touch (Saito et al., 2015).

3.1.4 Cutaneous wound healing process

A disruption of skin integrity immediately leads to the initiation of complex multi-dynamic cycles and reparative processes (Broughton et al., 2006; Reinke and Sorg, 2012). The time period it takes for a complete covering of a cutaneous defect varies from a view days in case of small incisional wounds and many weeks in case of open wounds after a complicated surgeries (Martin and Nunan, 2015). The temporal link between the main events during inflammation, proliferation and remodelling are summarised in **Figure 4**. Regaining tensile strength of the newly formed scar tissue usually takes a lot longer than the epithelial closure on the skin surface. After one year, a wound has regained about 80% of the origin tissue rigidity which is the reachable maximum (Gantwerker and Hom, 2011). Numerous more intrinsic and extrinsic factors determine the time to heal besides the dimension of the tissue defect. Intrinsic factors can be underlying diseases, for example: diabetes mellitus, circulatory disorders or the age of the patient and the location of the wound on the body. Extrinsic factors are constituted mainly by: temperature, medical treatments and the amount of pathogens of the direct environment (Guo and DiPietro, 2010). The healing process also differs depending on whether the defect heals by primary intention (sterile, straight wound edges adapted to each other) or secondary intention (open wound, non-adapted wound edges) (Sorg et al., 2017).

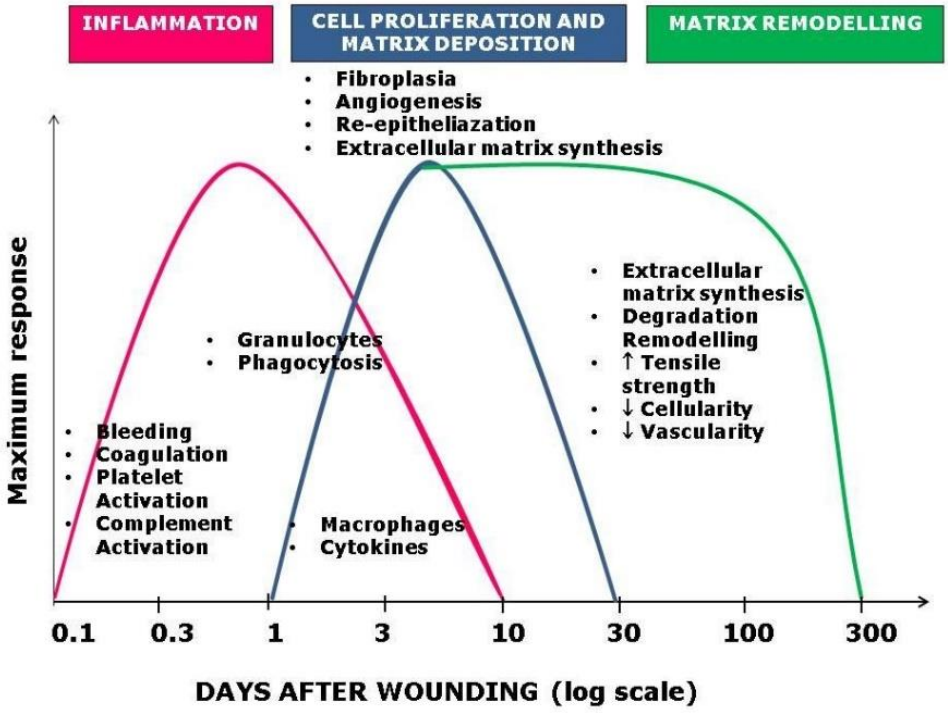

Figure 4 Timetable of the wound healing phases

Inflammation, proliferation and remodelling are timely overlapping. Main molecular and cellular features are listed chronologically in the certain stadium of the wound healing process. Inflammation and proliferation phase proceed in a classical curve progression with a peak. Remodelling phase proceeds in a steady state from day 10 until day 300 and thereby represents the longest phase. Taken from: https://www.fastbleep.com/biology-notes/8/278, day of access: 05.04.2018.

3.1.5 Inflammation phase

Inflammatory processes are initialised immediately after wounding (day 0), generally take up to 10 days and are defined as prolonged if they exceed a duration of three weeks (Gantwerker and Hom, 2011). **Figure 4** shows how by the time the inflammation phase has reached its “maximum response”, the proliferation phase already begins simultaneously. The five clinical indications for inflammation are redness, heat, pain, swelling and loss of function. Whenever skin becomes injured in subepidermal regions, the inner surface of blood and lymphatic vessels becomes exposed to the ECM. Inflammatory processes begin with haemostasis which reduces blood loss. Primary haemostasis is performed by the platelets forming an initial clot via the interaction of their GpIIB-IIIa receptor on the collagen of the damaged subendothelium to close the endothelial defect (Gantwerker and Hom, 2011). Secondary haemostasis occurs simultaneously and implies the activation of the intrinsic and extrinsic coagulation

cascade to form fibrin strands that stabilise the clot **(Figure 5)**. The entire clot consists of collagen, platelets, thrombin and fibronectin which release cytokines and GFs triggering the continuation of the inflammation phase (Broughton et al., 2006). Vasoconstriction of the vessel within close proximity of the wound, initialised by vasoactive substances (catecholamines and serotonin) and released by platelets are contributing to decrease the blood loss and the attachment of the clot to the wounded area (Broughton et al., 2006). Subsequently, vasodilatation of smaller vessels occurs, followed by invading neutrophils which are attracted by chemoactive substances: interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), interferon gamma (IFNγ) (Eming et al., 2007), complement components (e.g. C3a, C5a) and formyl-methionyl peptide products from bacteria and platelets (Enoch and Leaper, 2008). Neutrophils migrate from the blood vessel via diapedesis within the first 24 hours. They are responsible for clearing the wound from local bacteria, to degrade necrotic tissue by protease secretion and phagocytosis (Findikcioglu et al., 2012). By the time macrophages enter the wound which is usually within 48 to 72 hours after injury the second part of the inflammation phase is initiated (Velnar et al., 2009). Macrophages and other lymphocytic cells support the ongoing processes by continuing to dissolve contamination via phagocytosis and immunological functions as antigen-presenting cells. They release more chemokines, cytokines and GFs, for example transforming growth factor-beta (TGF-β), TGF-α, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) which initiate the next WH phase (Eming et al., 2007; Reinke and Sorg, 2012). There also is recent evidence that macrophages are crucial for WH processes and their depletion would lead to WH disorders (Bukowiecki et al., 2017) but beside neutrophils and macrophages, the essential function of mast cells has to be outlined. During the inflammation phase they affect the blood circulation via releasing histamine, a vasoactive substance and it was also investigated that they can change vessel permeability via the protease-activated receptor-2 (PAR2) (Theoharides et al., 2007). They also have antimicrobial effects (Metz et al., 2008) and can stimulate angiogenesis by releasing pro-angiogenic factors such as VEGF, fibroblast growth factor-2 (FGF-2), PDGF and interleukin-6 (IL-6) (Ammendola et al., 2013). The progress to the next WH stage of proliferation is also prepared by the release of serin-proteases (chymase and

tryptase) from the mast cells that limit the excessice ECM production (Ammendola et al., 2013).

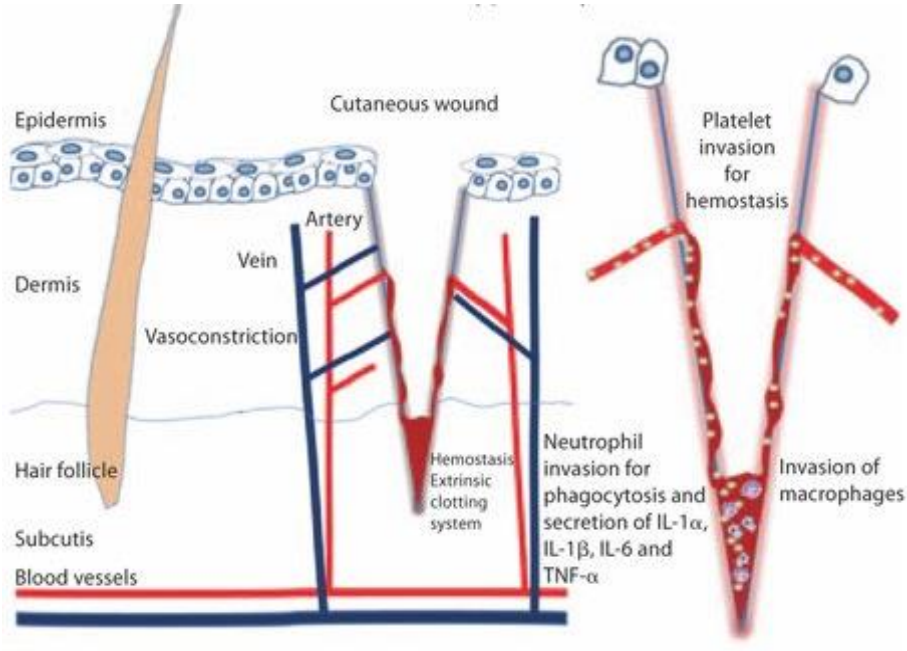

Figure 5 Haemostatic mechanisms and molecular signalling during the inflammation phase
Induced by a cutaneous cut, the reduction of blood loss continues with platelet invasion and haemostasis. The invasion of neutrophils and secretion of cytokines induces phagocytosis which leads to an attraction of macrophages. Taken from: https://www.researchgate.net/publication/229152941_Wound_Repair_and_Regeneration/figures?lo=1, day of access: 07.05.2018.

3.1.6 Proliferation phase

The proliferation phase starts timely overlapping with the inflammation phase. **Figure 4** shows that it lasts from day 1 until day 30 and that it is characterised by reepithelialisation, angiogenesis and synthesis of the ECM. During this phase the wound is covered by a layer of exudates containing GFs. Therefore, it requires a covering wound dressing to protect the surface of a wound for the uninterrupted completion of this phase. The restoration of a continuous epithelium is directed from the wound edges as well as from the ESC niches located in: the HF bulge, the base of sebaceous glands and in the BL of the interfollicular epidermis (IFE) (Martin and Nunan, 2015; Pastar et al., 2014). Under homeostatic conditions the ESCs are responsible for the cyclic renewal of the structure in accordance to their location but in case of an injury the stem cell function is convertible, for example stem cells from the IFE can differentiate to HF stem cells (Arwert et al., 2012).

Reepithelialisation starts with KC migration from the wound edges. To facilitate cell movement, the junctions of desmosomes between the cells and hemidesmosomes between KC and BL need to be dissolved. Transcriptional factor “slug” plays an important role in desmosomal disruption (Savagner et al., 2005). A restructuring of the adhesive molecules of the heimodesmosome integrin expressed in basal KC and LN of the lamina densa of the BL, occurs via switching from $\alpha 6\beta 4$ integrin to $\alpha 1\beta 3$ integrin for LN5 binding (Nguyen et al., 2000). The switch of integrin expression also leads to an adhesion of KCs to parts of the clot (vitronectin and fibrin) and to collagen of the subepidermal tissue (Falanga, 2005). Those migrating KCs can be identified by an upregulated expression of the keratins 6, 16 and 17. It was hypothesised that these changes of the cytoskeleton structure improve the viscoelastic properties of migrating KCs (Wong and Coulombe, 2003). Besides TGF-α and KGF, EGF is an indispensable GF for this WH phase (Pastar et al., 2014).

While KCs migrate from the wound edges, stem cells start to proliferate in the IFE. The ESC niches of sebaceous glands and HFs are also activated most notably by GF of the EGF family (Li et al., 2017).

Besides the development of a new epithelium, the ECM needs to be re-established. Stimulated by PDGF and EGF from the macrophages and platelets, fibroblasts start to migrate from the surrounding tissue and contribute to the formation of granulation tissue. The formation of granulation tissue defines WH by secondary intention, whereas primary wound closure proceeds by reepithelialisation. Granulation tissue consists of fibroblasts, newly formed vessels and immature collagen (type III) (Gantwerker and Hom, 2011). This provisional ECM also serves as a scaffold for cell adhesion and regulating movement, growth and differentiation of the included cells **(Figure 6)**.

Formation of new blood vessels to establish tissue perfusion is a crucial event of the WH process. It starts during haemostasis and continues until the wound is filled with newly generated granulation tissue (Singer and Clark, 1999). This process is most notably controlled by VEGF, PDGF, bFGF and the serin protease thrombin. These factors bind to their receptors on endothelial cells of surrounding intact vessels and activate intracellular signalling cascades. To prepare the proliferation and thereafter migration of the endothelial cells into the wound bed, a secretion of proteolytic enzymes which dissolve the BL is performed by the activated endothelial cells. This process is

also called as “sprouting” (Reinke and Sorg, 2012). Endothelial cells are orientated on superficial adhesion molecules, for example αvβ3 and αvβ5 integrin to enable further endothelial growth (Newman et al., 2011). If these integrins are not functioning or blocked by antibodies, angiogenesis is not working which is an important aspect for new treatment strategies regarding the recent cancer research (Weis and Cheresh, 2011). Endothelial cells build tubular channels and get linked by forming a vessel loop which will differentiate to arterioles and venoles. During this process they are able to release matrix metalloproteinases (MMPs) which eliminate the sourounding tissue so the endothelial cells can proliferate. During further wound closure the smaller inner vessels regress and become interconnected to a dermal vascular network of radially arranged vessels surrounding the wound (Reinke and Sorg, 2012).

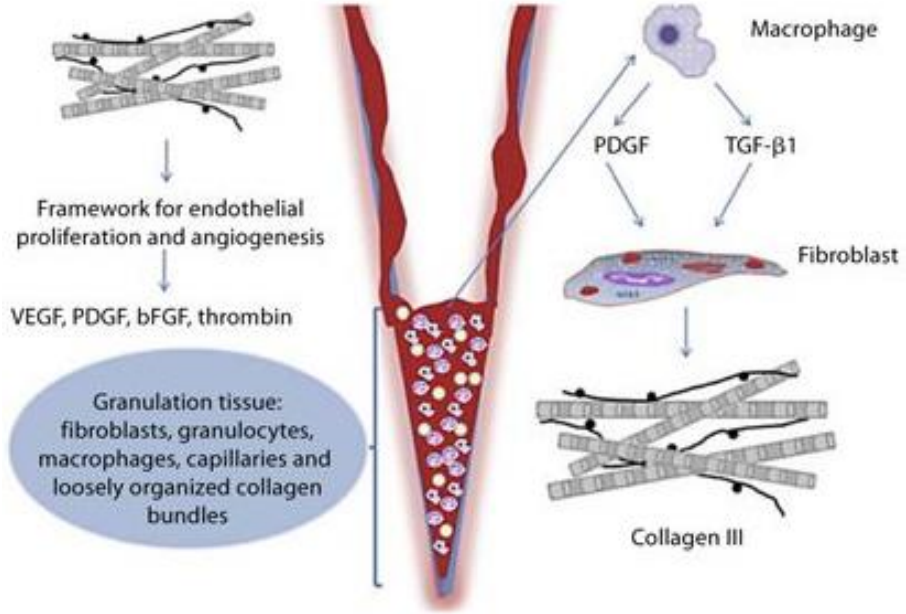

Figure 6 Formation of a scar tissue network during the proliferative phase
Granulation tissue continues to fill up the tissue defect and contains a framework for further endothelial proliferation and angiogenesis which is directed by many different growth factors. Taken from: https://www.researchgate.net/publication/229152941_Wound_Repair_and_Regeneration/figures?lo=1 day of access: 07.05.2018.

3.1.7 Remodelling phase

The remodelling is the last and longest phase of the WH cascade and can take more than one year. Synthesis and remodelling of the mature wound proceeds simultaneously and turns into a steady state, approximately 3 weeks post wounding (Gantwerker and Hom, 2011; Velnar et al., 2009). The definition of completed WH

implies that the wound tissue has become acellular and avascular (Greenhalgh, 1998). Cell types like macrophages and fibroblasts which were fundamental in the earlier WH phases switch to apoptosis. While the temporary scar tissue becomes rearranged and fibroblasts differentiate into myofibroblasts which have a contractile function and reduce the gap between the wound edges (Hinz, 2007), the replacement of type III collagen and provisional ECM matrix with type I collagen proceeds **(Figure 7)**. The amplified ratio of type I collagen is arranged in small parallel bundles reducing scar dimension and increasing tensile strength. Nevertheless, a scar will never gain the same tissue integrity like the original unwounded tissue (Son and Harijan, 2014). These last steps of WH are mainly directed by the MMPs secreted by fibroblasts, macrophages and endothelial cells and conducted by changing concentrations of TGF-β, PDGF, IL-1 and EGF (Broughton et al., 2006). The resultant scar differs from uninjured skin because some skin components will never recover completely, for example rete pegs which embed the connective tissue matrix stabilising the tight connection between epidermis and dermis. Additionally, subepidermal appendages like HFs or sweat glands will never become recovered completely (Reinke and Sorg, 2012).

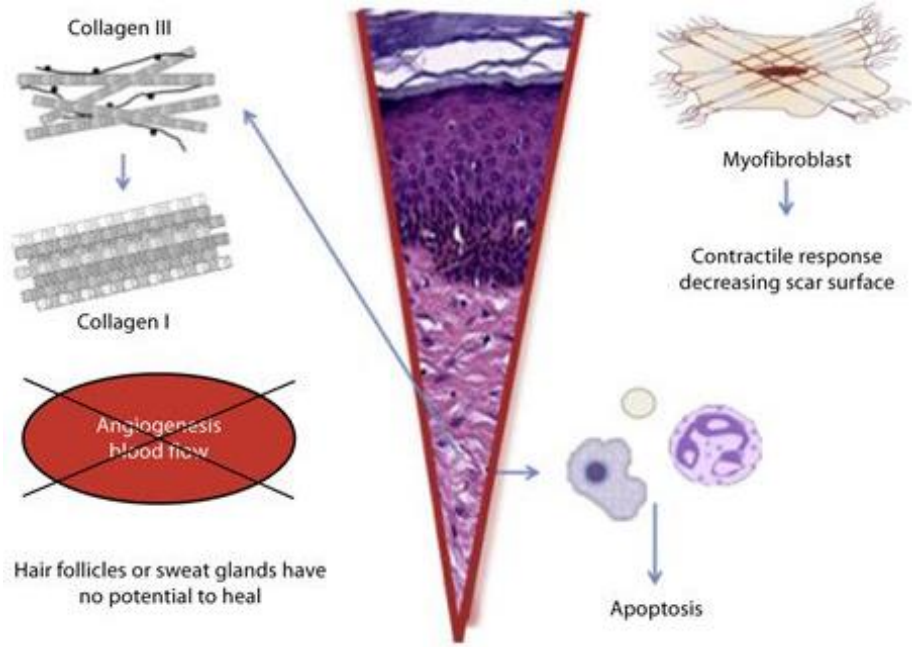

Figure 7 Final restructuring of the scar tissue during the remodelling phase

The final transforming steps of the temporary to the final scar tissue include the conversion of fibroblasts to myofibroblast, the termination of tissue production and the rearrangement of collagen from type I to type III. Taken from: https://www.researchgate.net/publication/229152941_Wound_Repair_and_Regeneration/figures?lo=1, day of access: 07.05.2018.

3.2 Chronic wound healing

A chronic wound develops if the above described phases fail to proceed in their cellular and molecular mechanisms and exceed the physiological duration of wound closure (Lazarus et al., 1994). Many chronic wounds show prolonged inflammation phases due to persistent infections and the disability of epidermal and dermal cells to respond to reparative signals (Demidova-Rice et al., 2012a).

The limit of time between acute and chronic WH differs depending on the literature. In the guidelines of the German *Arbeitsgemeinschaft der wissenschaftlichen medizinischen Fachgesellschaften* (AWMF) about the treatment of chronic wounds, a wound is defined as a chronic wound after a failure to reepithelialise the surface of the skin defect after 8 weeks (Burckhardt et al., 2012). Looking for a specific timely definition of chronic wounds many different statements were found, therefore an extract of those is listed in **Table 1** below.

„Als chronische Wunde werden alle Wunden bezeichnet, die nicht innerhalb einer physiologischen Abheilzeit von **2-3 Wochen** epithelisiert sind“	(Gillitzer, 2002)
„Slow healing is specified by defining a time frame (present for more than **4 weeks**) to seperate chronic ulcers from acute wounds”	(Mekkes et al., 2003)
„Chronic wounds are defined as wounds expected to take time to heal because of 1 or more factors delaying healing. Depending on the cause of the wound, wounds taking more than **4 to 6 weeks** to heal are considered to be chronic”	(Vaneau et al., 2007)
„A chronic wound is defined as a break in the skin of long duration (**>6 weeks**) or frequent recurrence”	(Fonder et al., 2008)
„Als chronisch wird eine Wunde eingestuft, wenn eine sekundär heilende Wunde trotz kausaler und sachgerechter lokaler Behandlung innerhalb von **3 Monaten** keine Tendenz zur Heilung zeigt bzw. nach **12 Monaten** nicht spontan abgeheilt ist“	(Dissemond et al., 2003)
“At the extreme end, chronic wounds – defined as a barrier defect that has not healed in **3 months** – have become a major therapeutic challenge throughout the Western world and will only increase as our populations advance in age, and with the increasing incidence of diabetes, obesity and vascular disorders”	(Martin and Nunan, 2015)

Table 1 Various citations about the timely definition of a chronic ulcer

A variety of statements about the lapse of time for healing durations which define a wound to be treated as a chronic wound. The time specifications are marked in bold letters and differ between 2 weeks and 12 months.

3.2.1 Epidemiology of chronic ulcers

Data which is based on the information of a statutory insurance showed that in Germany in 2012 the prevalence of treated chronic wounds was rated at 330,000 patients (standardised prevalence rate 0.4%). The incidence was estimated at 180,000 newly notified chronic wounds (standardised incidence rate 0.2%). Comparing the data from 2012 with the data of the preceding year 2010, a trend of increase is reflected (Heyer et al., 2016). In **Figure 8** the incidence rate of chronic ulcers is demonstrated, as well as the distribution of different kinds of ulcers and the growing occurrence depending on the age. Chronic wounds increase correlating to a higher age because of the growing incidence and prevalence of underlying diseases, for example the risk of getting the disease of diabetes mellitus for children who were born in the United States in the year 2000 amounts 32.8% for male patients and 38.5% for female patients (Narayan, 2003). A growing population in general, the demographic changes in the industrial countries and the fact that a higher age raises the risk of developing an ulcer contribute to the increased incidence of chronic ulcers in the future. Estimations have revealed that 1-2% of the world population will suffer under chronic WH at least once during lifetime (Eaglstein et al., 2012).

Additionally, chronic skin ulceration in an ageing population represents a complex clinical challenge and is associated with significant morbidity, not to mention the substantial economic costs (Alavi et al., 2016; Järbrink et al., 2017). Mean annual total costs for the treatment of a chronic leg ulcer in Germany were estimated with 9000 Euros per patient, per year. Taking into account that approximately 330,000 patients are actually treated, total costs have been estimated at 3 billion Euros which are spend on chronic wound care every year (Heyer et al., 2016).

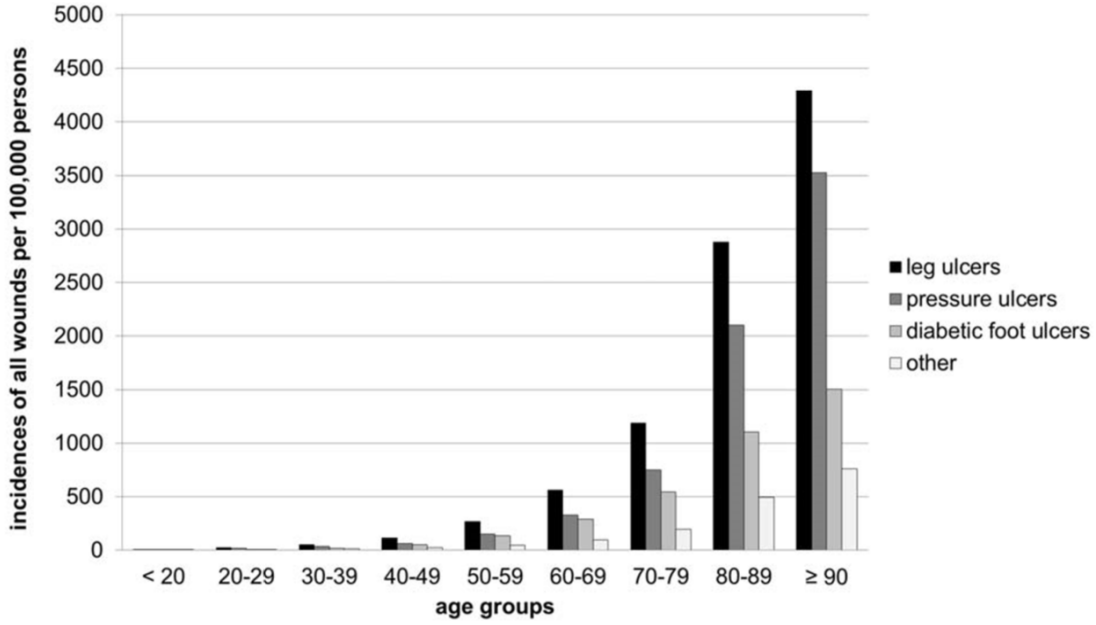

Figure 8 Incidence of a certain patient group with chronic wounds

The table shows the incidences of all wounds per 100,000 persons with the diagnosis of a chronic wound and a wound-relevant medical prescription, who were insured at BARMER GEK, in the time period of 2010-2012, on the y-axis. On the x-axis patient groups are divided per age decade in an increasing manner. Leg ulcers due to circulatory disorders are the largest group, followed by pressure ulcers, diabetic foot ulcers and other causes in a descending order. Taken from: Heyer et al., 2016.

3.2.2 Aetiology of chronic ulcers

Many different constellations of risk factors can possibly lead to a chronic wound. One of those is contamination by bacteria or differently caused debris. If a wound is constantly challenged by contamination and therefore highly activated inflammatory processes, neutrophil granulocytes and macrophages are not capable of continuing with further WH processes and cytokine signalling becomes out of balance (Grice and Segre, 2011). Another essential part of a successful WH process is the supplementation with sufficient nutrients and oxygen. Hence, cachexia because of malnutrition or a tumour disease also are risk factors for developing a chronic wound (Kavalukas and Barbul, 2011).

Basically, there are three major types of chronic wounds. They can be classified by their underlying diseases leading to a WH disorder: 1. venous or arterial circulatory disturbances, 2. diabetes and 3. constant mechanical pressure (Demidova-Rice et al., 2012a). Venous ulcers state the biggest part of chronic ulcers with more than 80%

(Montfrans et al., 2014). Reasons for venous insufficiency are: a long time of existing varicosis leading to angiodysplasia and unphysiological blood circulation, valvular insufficiency and secondary insufficiency as a postthrombotic syndrome. All of those reasons have in common that there is a constantly increased venous pressure.

An increased blood pressure inside the veins leads to a higher permeability resulting in baring plasma components and fibrin into the surrounding tissue which has a negative influence on WH by impeding synthesis of collagen, binding GFs and by forming precapillary fibrin cuffs (Walker, 1999). Those above described mechanisms are summarised in an established theory about the development of an ulcer and called the “fibrin cuff theory”. Another way of explaining the chronic ulcer formation is the theory about the chronic inflammation and unbalanced chemokines which was described in the first paragraph of this chapter and is also called “white blood cell theory” (Comerota and Lurie, 2015; Raffetto, 2013).

Patients with diabetes mellitus are at risk of developing a “diabetic foot syndrome”. Those poorly healing food ulcers develop because of polyneurpathically caused analgesia, which causes a decreased tactile and nociceptive perception leading to pressure induced ulcers. Polyneuropathy can also affect motoric nerve fibres and cause a Charcot’s foot which is a musculoskeletal malposition which also leads to pressure ulcers (Alavi et al., 2014). Further neuropathy entails microangiopathy due to the long-term increased glucose levels and an impaired muscle metabolism in the concerned tissue. In summary, the typical tissue features of chronic diabetic wounds are lower cell division potential, dysfunctions of cell-cell communications and the cell motility is diminished. Macroscopically, there are vascular changes of arteries and veins (Falanga, 2005).

Pressure ulcers are also associated with neuropathy due to a dysfunctional sensory perception. Immobility leads to a constant pressure which affects the underlying tissue with a decreased oxygen supply resulting in vascular ischemia and necrosis of the surrounding tissue (Defloor and Grypdonck, 1999). Finally, there are some additional subgroups of patients who have other reasons for delayed a WH or have a higher tendency of developing wounds like immunocompromised patients, for example long-term steroid therapy, Acquired Immune Deficiency Syndrome (AIDS), or organ-transplanted patients (Bootun, 2013). It can be summarised that there are certain groups of the population who are more likely to develop a chronic ulcer. People who

belong to this predisposed group are: at a higher age, smoker, diabetic, immobilised, suffering under a chronic (malignant) disease.

3.2.3 Current treatment of chronic ulcers

The treatment of the underlying diseases has highest priority as well as preventative measures to avoid the occurrence of new wounds. In case of circulatory disorders, interventions for revascularisation should be carefully evaluated, if it is an arterial circulatory disorder. Wounds caused by dysfunctions of drainage in the venous or lymphatic system could be prevented by specific compression treatments (for example compression bandages or stockings, frequently applied lymphatic drainages). It was also reported that a leg elevation above the heart level of 30 minutes several times per day can reduce leg oedema and improve microcirculation (Kirsner and Vivas, 2015).
A constant control of blood glucose levels from diabetic patients, the wearing of padded shoes and frequent pedicures avoid the formation of skin lesions (Braun et al., 2014).
Immobile patients who are bound to wheelchairs or beds need to receive pressure-redistributing support surfaces to decrease the interphase pressure (for example high-technology dynamic matrasses or overlays) to prevent decubital ulcers.
The main principle of the appropriate local treatment is to modify the wound dressing depending on the current WH phase and to keep it moist (for example with hydrogel or hydrocolloid and foam dressings) (Bedürftig and Eder, 2015). In the early stages of a wound, an increased exudation caused by inflammatory processes and bleeding requires a wound dressing which is capable of high absorbency (for example alginate, or superabsorbent polymers). According to the fact that persistent inflammatory processes caused by exceeding wound contamination can prolong WH processes, effective disinfectant agents are also necessary in the early WH stages. *In vitro*, silver can be either bactericidal and cytotoxic but the effectiveness of silver as an antibacterial component of wound dressings has not been approved yet (Rüttermann et al., 2013. The topical application of antibiotics is obsolete (Klein et al., 2007). In case of large and adhesive debris or a high production of devitalised tissue of the wound a surgical debridement is helpful to support healing processes (Wilcox et al., 2013). Also biosurgery with maggots has been reported to have a positive effect on wounds by promoting the formation of granulation tissue, shortening the time to heal and having a disinfectant effect (Wollina et al., 2001). In later stages of open sores, a wound

dressing needs to fulfil the requirement of non-traumatic coverage (for example, impregnated gauzes) and mechanical protection (for example, polyurethane foam, hydrocolloids).

If a wound remains unhealed without any satisfactory progress, despite applying all conventional treatment methods, a more specialised adjuvant wound dressing is needed. The field of tissue engineering represents biomaterials, acellular substances which are assembled with adhesion peptides that promote cell communication, fibroblasts, KCs or GFs and serve as temporary wound coverage (e.g. Apligraf®, Dermagraf® and Regranex®) (Veves et al., 2001). HP802-247, component of a fibrin spray, is also a tissue engineered product, which releases allogenic fibroblasts and KCs into an ulcer and was proven to significantly reduce wound area within 14 days (Kirsner et al., 2012).

Even though the “negative pressure wound therapy” is commonly applied in clinical practice, especially for the treatment of deeper cutaneous defects, reviews of the cochrane collaboration state that the effect is not sufficiently evidence based (Dumville et al., 2015).

Further physical treatment strategies are hyperbaric oxygen, electrical stimulation, application of magnetic or shock waves and ultrasound therapy. The hyperbaric oxygen therapy is an evidence based recommendation for the diabetic foot ulcer and the magnetic field therapy can be taken into consideration for the venous foot ulcer (Burckhardt et al., 2012; Kranke et al., 2015).

As a future perspective, the local treatment of chronic wounds with stem cells becomes further into the focus of WH studies. There are some promising approaches in the treatment by applying bone marrow – mesenchymal stem cells and also adipose tissue – mesenchymal stem cells (Amato et al., 2016).

Statements about WH promoting effects of honey or aloe vera are not sufficiently proved by studies yet (Jull et al., 2008).

3.3 Wound healing models

A number of WH models currently exist and can broadly be categorised into *in vivo* and *in vitro* models. The majority of WH research is performed in rodent models and *in vitro* (Wilhelm et al., 2017) even though the concordance of therapeutic effects in humans is 78% with those observed for pigs which is higher than in rodents (53%) and *in vitro* (57%) (Gordillo et al., 2013). Due to reasons like ethical limitations, comparability and reproducibility the usage of human skin in preclinical WH models represents a negligible factor despite its high significance for representing WH processes in clinical conditions.

3.3.1 Scratch assay

The scratch assay is a convenient model for studying WH (**Figure 9**). The technique is based on a single cell system consisting of a monolayer of the same cell type located on a plastic surface of culture dishes or other substrates, for example LN, collagen or vitronectin. Creating a gap within this monolayer which can be done mechanically with a plastic pipette, razor blades, a 23-gauge stub adapter, a 25-gauge needle in a standardized jig or a rotating silicone tip (Gottrup et al., 2000). A chemical method for inducing a disruption of the cells would be performed with alkali (Buisson et al., 1996) and other techniques which implement a teflon bar or a siliconized cloning cylinder which is removed after the first time of incubation and thereby induces a cell-cell interruption (Holzmann et al., 1994; Pilcher et al., 1997).

With this assay migratory and proliferative processes during WH of particular cell types can be investigated. If the focus is on studying cell migration, proliferation can also be eliminated by pre-treatment with a DNA synthesis inhibitor (Cha et al., 1996).

The Boyden chamber assay is a classic example for studying directional migration (chemotaxis) (Postlethwaite et al., 1976) and it is commercially available as a standardised research product. Furthermore, status of proliferation can be assessed, for example by immunodetection of proteins like Ki-67, or by using an electronic cell counter (Calderon et al., 1996; Sawhney and Hall, 1992). This assay also provides the opportunity to measure the biosynthetic activity in terms of collagen synthesis, or to determine the transcription for different proteins using Northern blotting (Gottrup et al., 2000).

A variety of different cell types and other tissue compartments (endothelium, dermis, appendages) which play an important role during physiological WH processes are missing (Koschwanez and Broadbent, 2011).

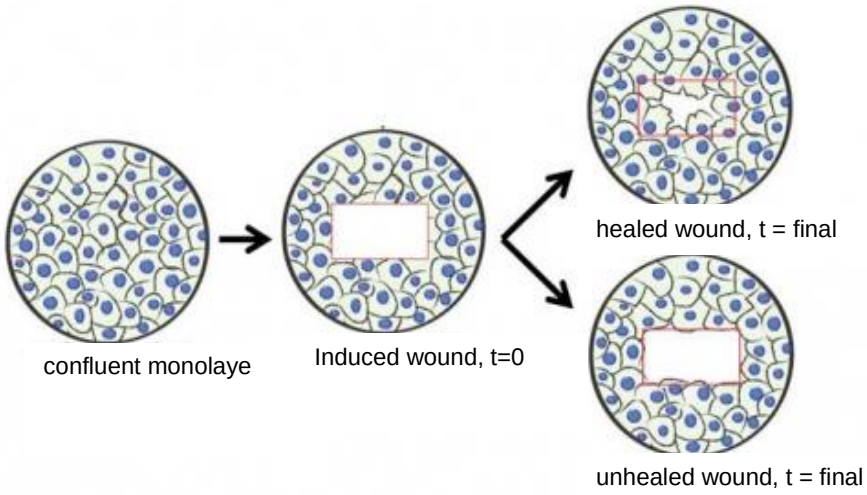

Figure 9 Experimental setup of a scratch assay
A confluent monolayer on a petri dish is “wounded” by artificial scratching in a reproducible manner. On the upper right, a layer with the “healed” defect after a certain time is displayed and on the lower right, a situation of failed wound closure is demonstrated.
Taken from: http://www.valasciences.com/services/migration-wound-healing, day of access: 08.05.2018.

3.3.2 Three-dimensional organ cultures

The three-dimensional (3D) organ culture model offers the opportunity to further investigate the interplay of different cell populations and the interactions of those with the ECM. This model is well-established and also purchasable as a convenience product (Matthes et al., 2014). In the classical setup of this model, fibroblasts are embedded into a collagen (usually type I) gel matrix. With the help of this experimental setup wound contraction was studied and the contribution of macrophages to wound contraction was demonstrated. The tension of the gel matrix was measured by an *in vitro* model of collagen contraction, which is called the culture force monitor (Gottrup et al., 2000). It has been shown that collagen production is decreased and collagenase activity is increased in fibroblasts which were integrated in the collagen gel compared to fibroblasts of the two dimensional cell systems. By this, apoptotic fibroblasts of

maturing granulation tissue *in vivo* can be reproduced and studied (Grinnell et al., 1999) and quantitative analyses of tissue remodelling events including signal transduction were investigated (Robinson et al., 2016). Because those early cell culture models revealed a short viability due to self-induced destruction, it has been optimised. The development of over 100 different types of matrices and scaffolds offer the opportunity to adapt the model depending on the type of the study (Ravi et al., 2015). A representative 3D model which enables studying regenerative epidermal processes *in vitro* for at least 24 weeks was developed with a specific fibroblast-derived matrix from normal human dermal fibroblasts that creates a complex human ECM and gets closer to the cutaneous conditions *in vivo* (Berning et al., 2015).

Although this model enables to examine many important cellular characteristics such as morphology, motility, fibroblast proliferation, response to pharmacological agents and protein production, the crucial role of skin appendages blood vessels and nerves is missing (Sriram et al., 2015).

3.3.3 Wound healing studies with animal skin

In vitro assays are indispensable for investigating isolated processes of the mechanisms during wound repair. For the involvement of all interactions and complex extracellular environment, it is also necessary to examine WH *in vivo*. Animal models are mostly provided by rodents, predominantly by mice and rats even though the porcine WH model would be more suitable to compare it to human WH (Wilhelm et al., 2017). Pigs and humans resemble in their cutaneous structure and mechanisms of WH. In contrast to the “loose” skin type of rodents and also partly (at the trunk) of cats, dogs and horses or ponies which can also be found while looking for larger animal WH models, the skin type is “tight”. The main mechanism of wound closure in pigs and humans is reepithelialisation instead of wound contraction (Volk and Bohling, 2013). Reasons against choosing the pig as a WH model are mainly the costlier, species-appropriate keeping and additionally they are larger, have a longer gestation time and afford more specialised staff than rodents. Furthermore, there are only a few transgenic pig lines available (Ansell et al., 2012). Whereas a variety of transgenic mouse models and knockout mice are available for studying the function of individual proteins and genes. Diabetic mouse models have contributed to investigate and understand the pathomechanisms behind delayed WH of diabetic patients (Fang and

Mustoe, 2008). Further animal models for chronic WH are listed in **Figure 10**. They are superior by including at least one of the three major metabolic disorders causing chronic ulcers (Nunan et al., 2014). WH models of other large animals are rare and normally do not include those ulcer-typical “pathological” conditions. In the field of stem cell therapy, there is a preliminary study about the successful therapy of equine degenerative joint disease with mesenchymal stem cells and platelet-rich plasma (Broeckx et al., 2015). In the field of basic WH research, all evolutionary stages (embryo, larva, pupal stage and adult fly) of the fly drosophila have been utilised for studying signalling pathways and genetic changes during wound repair (Razzell et al., 2011). Besides drosophila as a minimalistic organism, zebrafish larvae served to identify hydrogen peroxide (H_2O_2) as an early wound attractant after injury (Niethammer et al., 2009). Another zebrafish embryo model with induced mechanical vessel injury provided a system for studying vascular dysfunction and repair mechanisms in a whole animal model (Clay and Coughlin, 2015).

Amphibian skin has also been used for skin organ culture already a long time ago (Monnickendam and Balls, 1973; Yoshii et al., 2005). In a comparative *ex vivo* assay of human and frog skin the effect of thyrotropin releasing hormone (TRH) on WH was analysed and considered to have a positive effect on reepithelialisation (Meier et al., 2013).

Whilst animal models have the main advantage of studying WH in the whole organism setting, the experiments are expensive, time-consuming and require specific animal licenses and facilities compared to *in vitro* models. Furthermore, it is well known that the transfer of knowledge gained from animal models to humans is limited by the differences of the cutaneous structure and special WH mechanisms (Park et al., 2015).

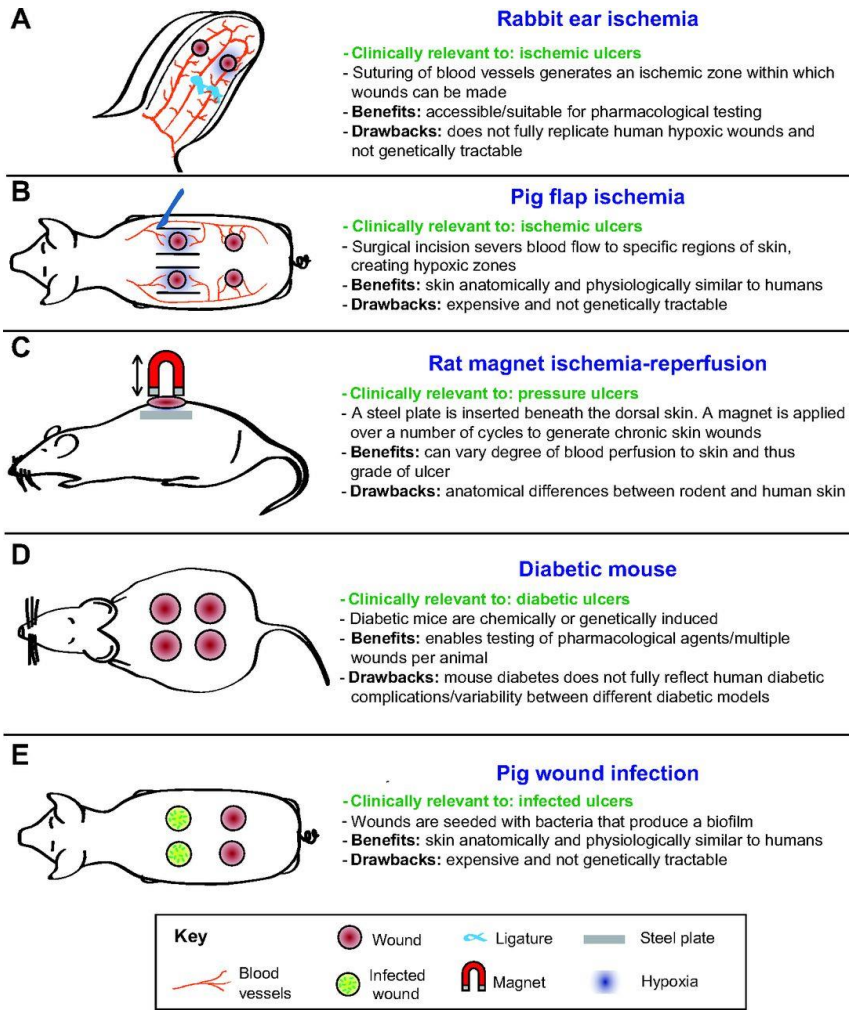

Figure 10 Animal chronic wound healing models

Five (A-E) animal models with the drafted experimental setup on the left side and the title, clinical relevancy and short description on the right. (B) is also applicable on rodents and rabbits, (C) also works with mice and (D) is practicable for rodents and rabbits, too. The legend below explains the drafts. Taken from: http://dmm.biologists.org/content/7/11/1205.figures-only, day of access: 07.05.2018.

3.3.4 Wound healing studies with human skin

For gaining most valid and significant knowledge about WH mechanisms and the optimal wound therapy, studies applying human skin provide the most transferable results. Executing WH experiments with humans (*in vivo)* is linked to many serious ethical barriers. Patients have to be informed consent about the risk of keloids, WH disorders or even systemic reactions like inflammation. Besides the medical risks, financial compensation for the test persons has to be considered. Established trauma methods to induce a wound are the “suction blister wound” by applying acids, by negative pressure (Kottner et al., 2013), heat application by laser (Ferraq et al., 2012;

Vits et al., 2015), cryotechnique with liquid nitrogen and surgical intervention of defined skin areas in course of another indicated surgery (Smith et al., 2015). The largest part of today's clinical studies about the treatment of chronic wounds is performed with patients suffering from chronic wounds. Those investigations comprise specialised single case studies and huge randomised controlled trials to test specialised wound dressings (Brölmann et al., 2013). Disadvantages of those retrospective clinical studies are high individual variations of health status, age, gender, time point of diagnosis and therefore a weak comparability and reproducibility. Evading this disadvantage and the strong ethical barrier of the *in vivo* human WH model with induced wounds, the *ex vivo* organ culture assay with human skin has to be taken into consideration. A human skin full-thickness organ culture model was developed by Moll *et al*.,1998. After plastic surgery a punch incision was pursued in the removed skin tissue. The emerged wound edges from the punch incision were used to evaluate reepithelialisation (Moll et al., 1998). This model has been further developed by modification of serum conditions, which extended the possible culture from the former maximum 7 days up to two weeks. This improvement was partly due to the replacement of foetal bovine serum to serum free conditions which made it also more comparable (Lu et al., 2007). A further variation of this model was the "punch-within-a-punch-model". Here, a smaller punch was added in the middle of the primary punch biopsy penetrating only the upper layers with a maximum depth until the dermis (**Figure 11**). This modification enabled the opportunity of analysing two more wound edges with underlying dermal tissue. The "punch in a punch" model was successfully applied to examine potentially WH promoting factors like thyrotropin releasing hormone (TRH) and thyroxine (T4) (Bodó et al., 2010; Meier et al., 2013; med. diss., Zhang, 2013). The advantages of this WH model are: a convenient technical handling, reproducibility and the use of human skin. Obviously, an *ex vivo* model is not able to display how WH mechanisms are influenced by inflammation, innervation or perfusion inside a complete organism. In summary, the choice for the optimal WH model depends on many factors and it has to be decided carefully depending on the intention of the study.

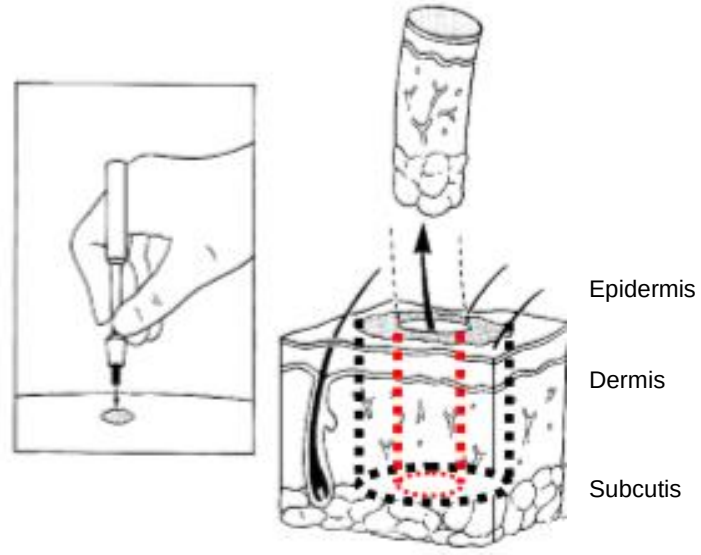

Figure 11 Punch biopsy organ culture model with human skin
The wounding of this organ culture model is performed with a punch biopsy. The black dotted line inside the tissue marks the outer punch edges, which is analysed in the “standard” organ culture punch model. In the “punch-within-a-punch” model two additional wound edges are induced by another smaller punch biopsy which is set inside the original punch (red dotted line). Taken from: http://northlandent.Blogspot.com/2012/06/how-to-do-punch-biopsy.html, day of access: 10.05.2018.

3.4 Thyroid hormones in wound healing

Human skin is one of the target structures of THs (Paus, 2010; Slominski et al., 2002). Patients with an underlying thyroid disease reveal certain physical changes. Xerosis cutis, myxoedema, thickened and brittle hair and nails and alopecia are typical symptoms of hypothyroidism. Hyperhidrosis, soft and fine hair, the phenomenon of the plummer’s nail (concave contour with distal onycholysis) and also alopecia are characteristics of hyperthyroidism (Heymann, 1992; Messenger, 2000; Parving et al., 1979). Hence, the constitution of skin and skin appendages depends on TH serum levels, a correlation between the thyroid status and WH processes becomes assumable and was been described before. In 1974, Mehregan and Zamick already

reported that systemic (triiodothyronine) T3 substitution accelerated WH and also improved the quality of wounds in euthyroid rats (Mehregan and Zamick, 1974; Safer, 2013). Also oral T4 accelerated the healing process of abdominal incisional wounds in hypothyroid rats (Erdoğan et al., 1999). Local TH treatment was also tested. The application of T3 on full thickness wounds on the back of mice resulted in better wound closure compared to only vehicle treated mice (Safer et al., 2005). It was already demonstrated that T3 promotes WH in guinea pigs whose (thyroid hormone receptor) TH-R have a close homology between TH-Rs in human. Here, the wound closure was accelerated most likely via wound contraction (Kassem et al., 2012). It was also shown that hypothyroidism has a bad influence on WH in humans. Patients with pharyngeal fistulas on a hypothyroid status showed WH disorders which could have been treated by substitution of THs (Alexander et al., 1982; Talmi et al., 1989). In a cohort study of Lindenbaum *et al.* the use of T4 combined with insulin, growth hormone, transferrin and sodium selenite inside a serum free medium as a topical wound dressing was applied on chronic ulcers and lead to a significantly better healing (Lindenbaum et al., 2001).

3.4.1 Hypothalamic-pituitary-thyroid axis and skin connection

Changes in thyroid metabolism result in skin alterations by the direct effect of TH, mediated via TH-Rs being present in the skin tissue and HFs (Contreras-Jurado et al., 2014; Safer, 2013).

Further explanations of the effect of TH on skin, all of the associated factors of the hypothalamic-pituitary-thyroid (HPT) axis and their cutaneous connection need to be outlined. TRH is expressed in dermal and follicular fibroblasts, neonatal KCs and melanoma cells *in vitro.* Additionally, human scalp follicles express TRH messenger ribonucleic acid (mRNA) *in vivo* (Slominski et al., 2002). TRH stimulates hair shaft formation, hair matrix KC proliferation, suppresses apoptosis of hair matrix KCs and prolongs active hair growth in human HF organ culture. It can also stimulate thyroid-stimulating hormone (TSH) expression and promote wound reepithelialisation in frog and human skin *in vitro* (Bodó et al., 2010; Gáspár et al., 2011; Meier et al., 2013). The TRH receptor (TRH-R) is also expressed in human scalp HFs on mRNA and protein level *in vivo* (Slominski et al., 2005, 2002).

TSH is expressed in human epidermis (Gáspár et al., 2010) and there is evidence that TSH controls KC energy metabolism and mitochondrial functions (Knuever et al., 2012). TSH also stimulates cytokeratin (CK) 5 and CK14 expression in the epidermis of human skin (Bodó et al., 2010). For the TSH receptor (TSH-R) it has been proven that its mRNA is expressed in cultured KCs, epidermal melanocytes and melanoma cells. Concerning the TSH-R expression *in vivo,* there is evidence for the expression on gene and protein level in human skin within the mesenchyme (Bodó et al., 2009) and also for the expression in the epidermis in non-scalp human skin (Cianfarani et al., 2010). A link between TSH-R expression in the skin by autoantibodies might play an important role in cutaneous autoimmune diseases and it was also described that TSH-R stimulation by systemic TSH promotes proliferation of human epidermal KCs and dermal fibroblasts (Bodó et al., 2009; Paus, 2010). Deoinidase D_2 and D_3 are peroxidise enzymes which induce activation or deactivation of THs. It was reported that they can convert T4 to T3 in human skin. Their gene expression was proven to take place in the majority of human epidermal and dermal cells, *in vitro* (Slominski et al., 2002; Tiede et al., 2009; van Beek et al., 2008).

3.4.2 Structure and function of thyroid hormones

T3 and T4 are key players of the human energy metabolism (Boelen et al., 2012; Johannsen et al., 2012). The amino acid tyrosine is the basic structure for T4 and its metabolite. T4 holds 4 iodine (I_2) atoms and T3 is the most important active metabolite holding 3 (I_2) atoms. The conversion of T4 to T3 is performed by deionidases mainly in liver and kidney (Gu et al., 2007; Molina, 2013). The percentages of active free serum concentration are 0.3 % for T3 and 0.03 % for T4. The biological half-life time for T3 is 19 hours and 190 hours for T4. Biosynthesis and secretion are regulated by a complex control circuit demonstrated in **Figure 12**. On the top of this control circle TRH is activating TSH which is produced in the anterior pituitary. TSH stimulates biosynthesis and secretion of T4 and T3. The circuit is completed by the negative feedback regulation of the hormone production. An increased concentration of TH in the serum results in inhibiting mechanisms of the TRH and TSH release. The majority of TH in the serum is inactive and bound to carrier proteins: thyroid-binding globulin (TBG), transthyretin (TTR or prealbumin) and albumin (Gu et al., 2007). If it is required, T4 can be converted to T3 via the deionidase enzyme in each target organ. There are

three types of deionidases (D1, D2 and D3). D1 and D2 have an activating function by deiodinating T4, via removal of an iodine atom at the outer ring which results in the active T3 (St Germain et al., 2009). D3 functions as an inactivating enzyme for T3 (Bianco and Kim, 2006).

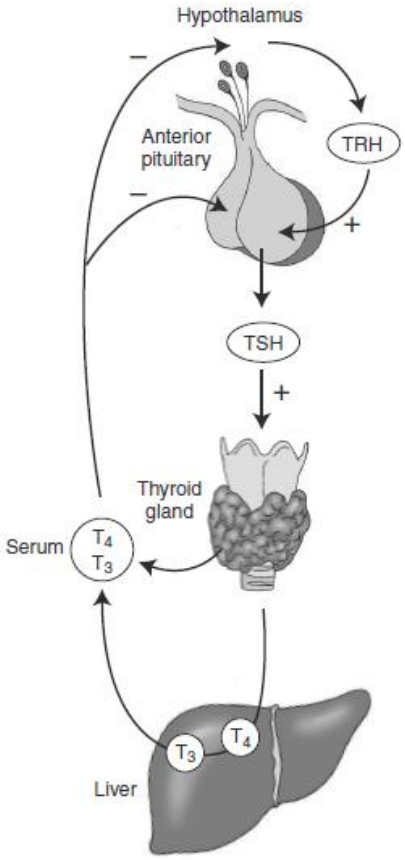

Figure 12 The circuit loop of regulation for thyroid hormone synthesis
Thyrotropin releasing hormone (TRH) is stimulated by low serum concentrations of the thyroid hormones (THs) triiodthyronine (T3) and thyroxine (T4) or central stimuli like stress, cold or circadian rhythm. It is released by the hypothalamus and directly stimulates the posterior pituitary gland which secretes thyroid-stimulating hormone (TSH) that activates the thyroid gland to produce more TH. A low serum concentration can also directly activate the anterior pituitary part to induce TSH production. Taken from: http://edusanjalbiochemist.blogspot.de/2012/12/how-thyroid-hormone-synthesis-and.html, day of access: 22.09.2017.

3.4.3 Biosynthesis of thyroid hormones

Biosynthesis of TH takes place inside the thyroid follicular cells and follicle colloid. The follicular cells synthesise and release thyroglobulin. Meanwhile, the uptake of iodide (I^-) is performed from the blood into the follicular cell, via the sodium-iodide symporter (NIS) which is located at the basolateral membrane. I^- passes through the cell and is transferred to the follicle colloid via the transport protein pendrin. Here, I^- becomes oxidised by the thyroid peroxidase (TPO) and is stored as I_2 (iodination). The glycoprotein complex thyroglobulin contains tyrosine components and is responsible for linking tyrosine to I_2 which results in mono- and diiodtyrosine, again via the enzyme, TPO (Brent, 2012; van Beek et al., 2008; Warner and Mittag, 2012). The linkage of I_2 bound tyrosine residues results in T3 and T4. Hormones are stored as macromolecular precursors and stay in the colloid bound to thyroglobulin and secreted from thyroid cells in a TSH dependent manner. **Figure 13** provides an overview of the way of TH synthesis.

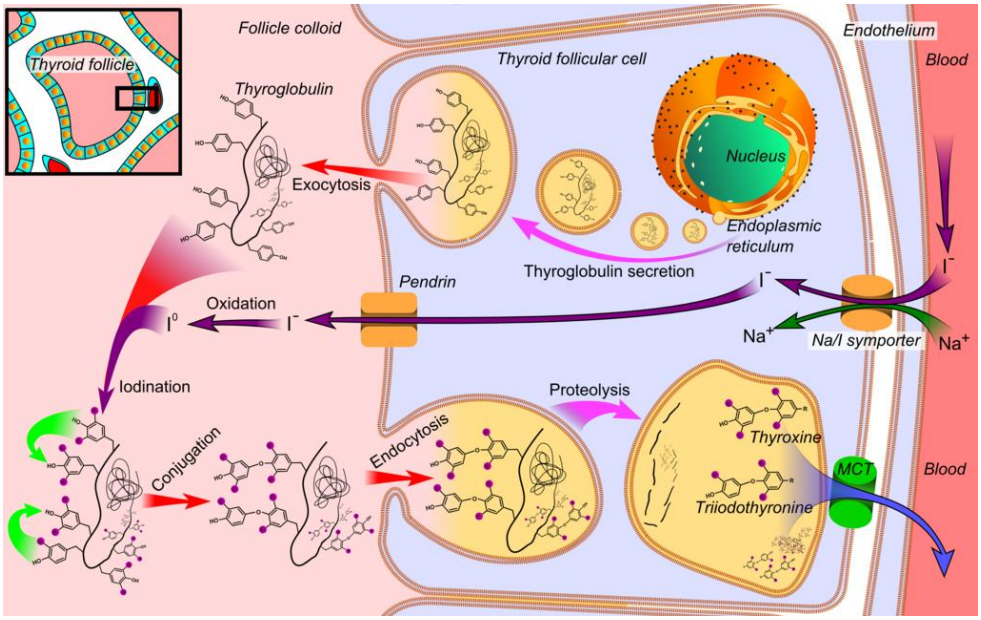

Figure 13 Biosynthesis of thyroid hormones

For thyroid hormone production the thyroid gland essentially requires sufficient iodide uptake from the serum and tyrosine molecules. The whole process can be summarised as: 1. uptake of the basic elements, 2. assembly of the hormone with the interactions of thyroglobulin and thyroid peroxidase and 3. liberation of the hormones into the blood. Taken from: https://commons.wikimedia.org/wiki/File: Thyroid_hormone_synthesis.png, day of access: 18.09.2017.

3.5 Thyroxine in clinical medicine

Levothyroxine, or L-thyroxin is the synthesised substance of T4 and one of the most commonly prescribed drugs in clinical medicine. In 2016 it was the second most prescribed medical drug in Germany, after ibuprofen on first rank (Schwabe et al., 2017). Indications are mainly hypothyroidism, treatment or prevention of euthyroid goiter, myxoedema coma and TSH suppression in well-differentiated thyroid cancer and thyroid nodules (Shoemaker et al., 2012). L-thyroxine is applied orally 30 minutes before breakfast to increase the intestine resorption. In a case of hypothyroid coma even intravenous administration can become indicated. Substitution therapy with T4 has been applied for a long time. First recognition of hypothyroidism as a disease syndrome was in the 1870s and the treatment was performed right away with extract of animal thyroid. Synthetic thyroid replacement therapy was performed since 1927 (Mitchell et al., 2009). In conclusion, the treatment with T4 has a long tradition in clinical practice.

The topical application of THs is not common in clinical drug therapy yet. There are approaches of clinical use by treating alopecia areata with a T3 ointment which was well tolerable but did not enhance hair growth (Nasiri et al., 2012). A clinical report about a treatment strategy of chronic wounds which contained an enriched cell culture medium including growth hormone, insulin and T4 showed an effective healing rate with complete wound closure of 65.6% and no intolerance reactions (Lindenbaum et al., 2001).

Summarising the facts that T4 has long been applied in clinical medicine and is a cost-efficient drug with a defined toxicological profile for clinical use (Biondi and Wartofsky, 2012; Brenta et al., 2007), makes it become a reasonable subject of research to investigate further potential use and thereby enhancing future therapeutic opportunities.

4 Material and Methods

4.1 Human skin wound healing model

Close to the experimental setup of Lu et al., 2007 and Knuever et al., 2012, we utilised a 4 mm punch of hair bearing, full-thickness human skin including the subcutaneous fat. This setup was fast reproducible and easier to handle than the “punch-within-a-punch” model (Meier et al., 2013; Moll et al., 1998)

4.1.1 Human skin samples and ethics approval

Temporal and occipital scalp skin samples were received as side products from cosmetic surgical procedures. All patients were female and at the age of 52-65 years, averaging 58 years and healthy. The patient demographics are listed in **Table 2**. As it is defined in the ethical agreement, skin sample recruitment had to be anonymised. Only the age, sex and skin sample location were specified.

Skin sample number	Sex	Age	Region
HS 12-106	Female	57 years	Face, temporal
HS 12-108	Female	52 years	Face, temporal
HS 12-147	Female	65 years	Face, frontal
HS 12-150	Female	58 years	Head, occipital
HS 13-050	Female	54 years	Head, occipital
HS 13-090	Female	62 years	Face, temporal

Table 2 Patient data of skin tissue donors

This table provides an overview of the origin of the skin which was treated and analysed in the experiments of this thesis. The skin sample number is composed of the first two letters “HS” which means “human skin”. The first two numbers are representing the year when plastic surgery was executed. The last three numbers are sequential numbering for labelling tissue samples arriving in the laboratory.

All patients provided informed consent and the study was approved by the Institutional Research Ethics Committee at the University of Lübeck (University of Lübeck, license: Reference 06-109). We sustained the scalp skin anonymously from patients who

underwent plastic surgery. The experiments were performed according to Helsinki Declaration principles.

4.1.2 Performing a full-thickness human skin organ culture

After surgical intervention the skin was delivered via overnight transportation to the laboratory. During the transport the tissue was cooled (4ºC) and stored in covered plastic test tubes filled with serum-free William's E medium (Lu et al., 2007). In the laboratory, skin samples were placed into a petri dish with sterile forceps and then "wounded" by a 4 mm punch biopsy and scissors. Skin parts showing macroscopic indications of tissue damage were separated. Subcutaneous fat and hair-bearing skin were retained (See **Figure 14**).

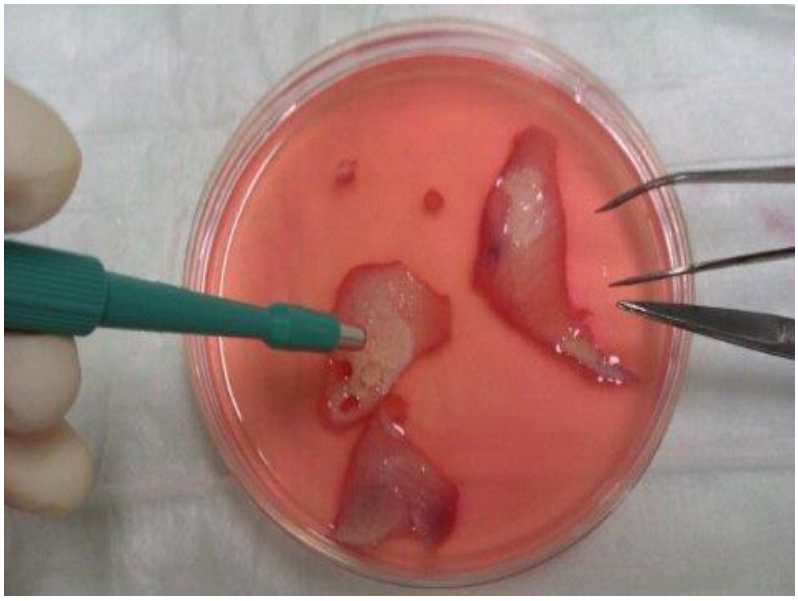

Figure 14 Preparation of the punch biopsies

Example of the extraction of 4 mm punch biopsie in William's E medium under sterile conditions.

The punch biopsies were placed into serum-free William's E medium which was supplemented by L-glutamine (2 mmol/l), hydrocortisone (10 ng/ml), insulin (10 µg/ml) and streptomycin (10 µg/ml)/penicillin G (100 IU/ml) (Lu et al., 2007) and served as the basic culture medium, left unmodified for the standard WH condition group (control group). All punches were cultured in a 6-well plate for three days (**Figure 15**).

Figure 15 Organ culture setup

Example of a 6-well plate with two skin punches floating at the air-liquid interface in 3 ml medium per well. Four punches of two wells were exposed to the same tissue conditions for three days.

At day zero, 3 ml of the culture medium and two skin punches which floated at the air-liquid interface were placed into one well. The medium was not changed during the culture period. A constant atmospheric pressure was kept automatically by the CO_2 incubator. At day three, all skin biopsies were removed from the medium. Two punches of each well were embedded in Shandon Cryomatrix and snap frozen in liquid nitrogen. The other two punches of the same condition were fixed in paraformaldehyde and then embedded in paraffin. Cryo punches were stored at -80ºC. The paraffin punches were stored at room temperature and were not used for further analysis. To prepare the appropriate staining, the cryo punches were cut at -21°C to -23°C into longitudinal sections (7 µm thickness) with the cryostat and absorbed on a microscopic slide. Afterwards, the slides were stored again at -80°C until they were used for (immuno-) histochemical studies and analyses.

4.1.3 Establishment of a “pathological” wound healing model

Glucose concentration, oxygen percentage and the amount of H_2O_2 were mostly based on previous pilot experiments conducted by our research team (data not shown). **Figure 16** shows an overview of the pilot experiments determining the appropriate experimental setup for “pathological” conditions.

A concentration of 138.8 mM (2,500 mg/dl) of glucose was applied to create hyperglycaemic circumstances. In previous pilot experiments glucose concentrations of 55.5 mM (1000 mg/dl) and 555.5 mM (10000 mg/dl) had been applied as well. There was no cell viability at the concentration of 555.5 mM and no objectifiable difference to WH processes of skin punches under 55.5 mM. This lead to the conclusion of utilising 138.8 mM for this thesis. Since the diagnosis of Diabetes mellitus is defined as a fasting serum glucose of ≥ 7 mmol/l (≥ 126 mg/dl), or the occasional serum glucose is ≥ 11.1 mmol/dl (≥ 200 mg/dl), an amount of 138.8 mM glucose is 10 times higher than the value of a “pathological” spontaneous serum glucose level (Herold, 2013). Diabetic tissue damages are long-term defects and therefore we had to accelerate the effect by a higher concentration. For adding glucose to the medium, we used D-glucose powder and solved the corresponding concentration of glucose under aseptic conditions in 10 ml William’s E medium using a vortexer.

Inducing hypoxia was initially started in the pilot experiments by adding cobalt(II)-chloride ($CoCl_2$) to the medium. According to literature research, CoCl2 leads to intracellular reactive species accumulation and disruption of mitochondrial membrane potential (Lee et al., 2013). It also causes an increase of erythropoetin (EPO) production by the activation of hypoxia inducible factor-1 alpha (HIF-1α). HIF-1α is a transcriptional factor which regulates the oxygen supply of a cell by the oxidation state of its subunit. It is inactivated in standard oxygen conditions (oxygen content of 20%) and activated under hypoxic conditions and induces EPO production, angiogenesis, cell-proliferation, inhibition of apoptosis and glycolysis. This leads to the conclusion that HIF-1α induction finally results in anti-hypoxic mechanisms (Ge et al., 2012). The next step for inducing hypoxia to create “pathological” conditions in our pilot experiments was to run organ cultures in a hypoxic incubator. With the setting of oxygen supply on 1% there were hardly any nuclei detectable. For finding our final experimental setup, we decided to induce oxygen supply of 5 % which is based on the results of the first above described pilot experiments and furthermore in conformity with a clinical example. Peripheral arterial vascular disease leads to an oxygen deficiency in the supplying tissue. The partial oxygen pressure in the tissue of a healthy human averages 80 mmHg (+/- 9.6 mmHg). In contrast, a patient suffering peripheral arterial disease, stadium III – IV, will show partial oxygen pressures of about 35 mmHg (+/- 19.8 mmHg) (Arastéh, 2018). The relevance of measuring transcutaneous oxygen

tension to detect the hypoxia and state of the vessel disease has been described before (Lusiani et al., 1988).

Oxidative stress was included as the third “pathological” factor which is increased in chronic ulcers and disturbs the healing process (Eming et al., 2009; Gould et al., 2015). To induce oxidative stress H_2O_2 was added to the medium. Within the first pilot experiments, a concentration of 250 mM again showed a severe tissue destruction and lead to a reduction of the concentration for the final experimental setup. 100 mM H_2O_2 was added in the appropriate concentration to each well

The amount of 3.06 µl of H_2O_2 had to be added twice daily after a time period of six hours to the “pathological” medium because of its volatile character. The fourth “pathological” impact was set by the withdrawal of insulin. Many diabetic patients suffer under WH disorders due to the metabolic changes with the lack of insulin playing a central role (Berlanga-Acosta et al., 2013; Mekkes et al., 2003). Therefore, the usual step of adding insulin to the culture medium was skipped for the preparation of the “pathological” medium. In summary, the “pathological” medium consisted of: William’s E medium (2.98 ml), L-glutamine (2 mmol/l), hydrocortisone (10 ng/ml), streptomycin (10 µg/ml)/penicillin G (100 IU/ml), lucose (138.8 mM), H_2O_2 (100 mM) under hypoxic conditions with an oxygen supply of 5%.

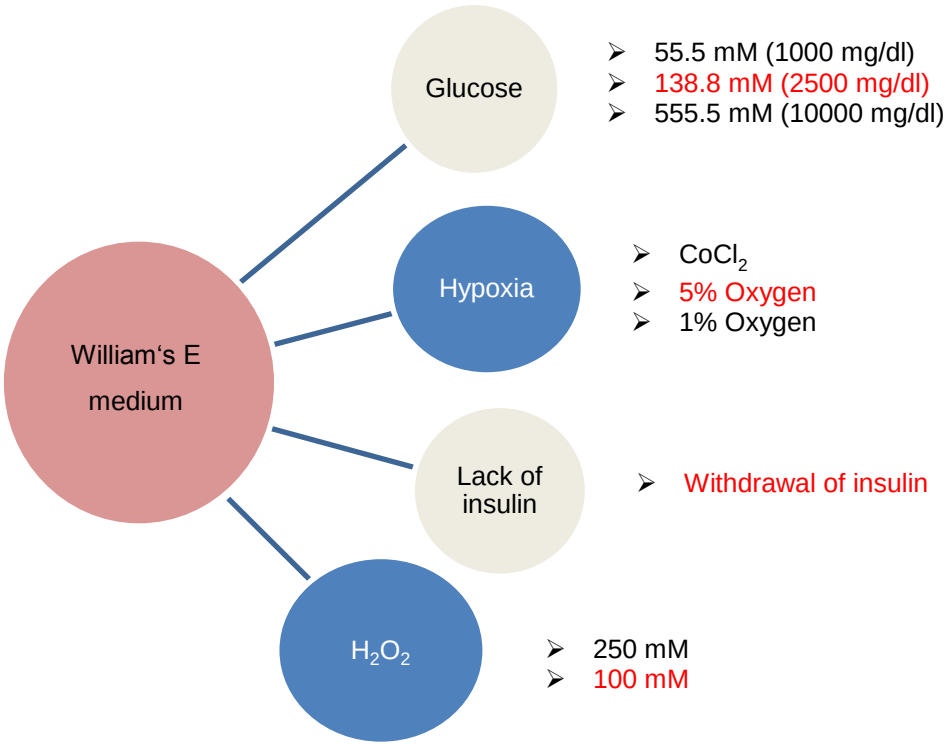

Figure 16 Constellations of "pathological" medium conditions

Different concentrations of the four "pathological" factors were combined with the William's E medium. Glucose was applied in three concentrations, hypoxia was induced by the incubator and by adding cobalt(II)-chloride ($CoCl_2$), insulin which is a standard component of the culture medium was withdrawn and hydrogen peroxide (H_2O_2) was added in two different medium conditions). The final setup, which was applied and evaluated in this thesis is demonstrated in red colour.

4.1.4 Thyroxine induced wound healing model

In the second part of this thesis, both organ culture models were also investigated after the supplementation of 100 nM T4. This concentration was tested in a previous project of our lab in which the influence of T4 on WH was tested. It was shown that T4 has a positive effect on reepithelialisation, KC migration, intracutaneous angiogenesis and upregulation of CK6 and CK15. The test samples cultured under 100 nM showed the most significant effects, which made us decide to test only the concentration of 100 nM in this thesis.

In the first part standard and induced "pathological" conditions were compared to create a "pathological" WH model. In the second part the "pathological" model was

applied to test T4 as a candidate WH promoter under “pathological” conditions. Those two separate experimental setups are demonstrated in **Figure 17**.

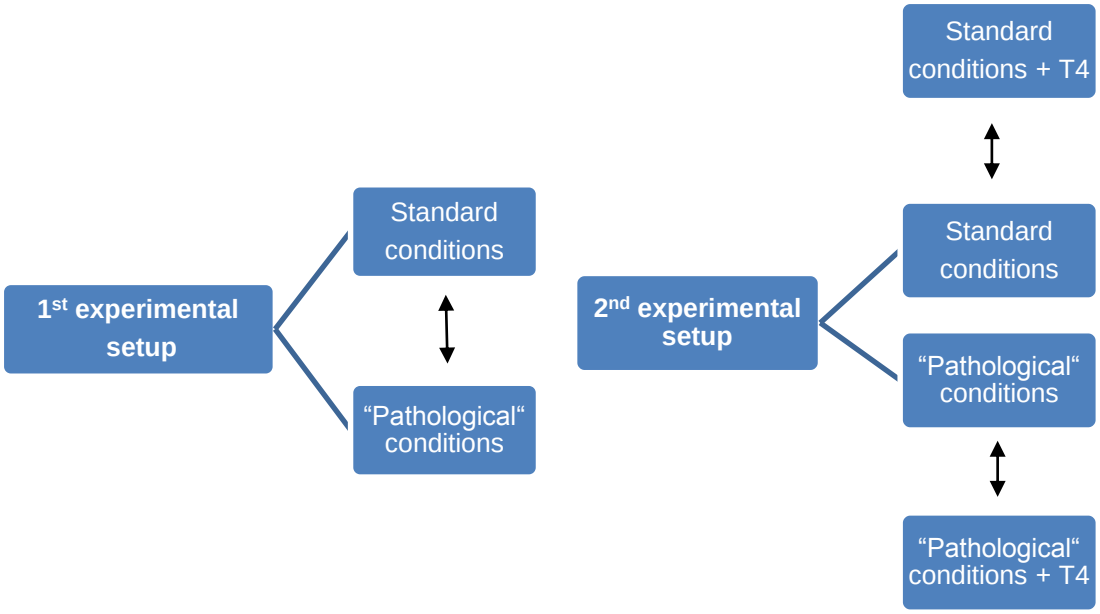

Figure 17 Study designs

An overview of the two experimental setups is show. In the first experimental setup (on the left), standard and “pathological” conditions were evaluated. Based on these results, the second experimental setup (on the right) provided to investigate the effect of thyroxine supplementation to the standard and “pathological” medium conditions.

4.2 Histochemical analyses

Histochemical stainings were performed using haematoxylin and eosin (H&E) stain and periodic acid–schiff (PAS) stain. Both of them are commonly used to visualise crucial structures of the epidermis. The principle of H&E stain shows KCs by their basic cell structure elements as it lets the nucleus appear purple/blue and the cytoplasm in red. PAS staining was applied to highlight the BL. Due to its amount of glycoproteins the BL appears in magenta red by the oxidation of the glycol groups with periodic acid resulting in two neighboured aldehyde groups which get chromophore properties when binding to the Schiff reagent.

4.2.1 Assessment of dyskeratosis using haematoxylin and eosin staining

For the assessment of dyskeratosis, slides were stained by the counter stain of Mayer's haemalum which is an oxidised product of haematoxylin and 0.1% eosin. At first, all slides were dried at room temperature for ten minutes. Afterwards a fixation in acetone for another ten minutes at -20°C took place. All slides were washed by distilled water for three minutes and were then stained by haemalum for 15 minutes. The slides were washed again under running tap water for 15 minutes and adjacent coated by eosin for one minute. To finish the process all slides were washed by alcohol solution in ascending sequence up to pure alcohol (alcohol 70%, alcohol 96%, alcohol 100%). Finally, the slides were put into xylol solution for ten minutes. The cover glasses were laid and fixed on the slides with Eukitt® solution. After drying under the laminar airflow for 12 hours they were ready to be analysed.

All cells between *stratum corneum* and *stratum basale* with the morphological features of premature keratinisation, which means a pyknotic, hyperpigmented, dense nucleus and lightened cytoplasm (personal discussion with Dr. C. Rose) were counted. Black arrows in **Figure 18** mark dyskeratotic KCs which were counted per section, per visual field.

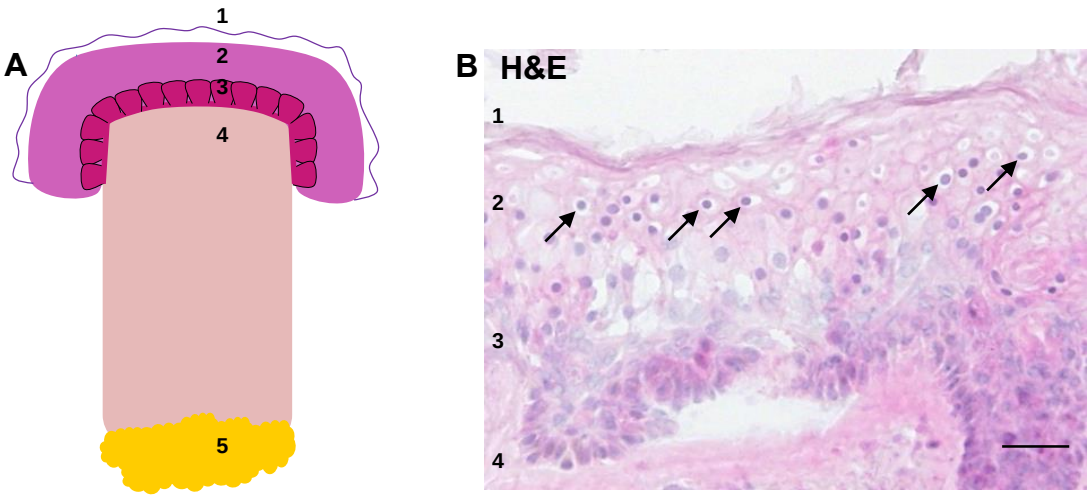

Figure 18 Assessment of dyskeratosis

(**A**) Schematic drawing of a punch showing labelled cutaneous layers (1-5). Assessment of dyskeratosis was performed in the granular and spinous layer, marked with 2. (**B**) Haematoxylin and eosin stained section of skin under "pathological" medium conditions showing dyskeratotic keratinocytes (black arrows) and cutaneous layers 1-4. 1 = cornified layer, 2 = granular and spinous layer 3 = basal lamina, 4 = dermis, 5 = subcutis. Scale bar: 50 µm.

4.2.2 Epithelial tongue and split formation evaluation using periodic acid Schiff-staining

The PAS reaction highlights the BL of stained skin sections in a purple-magenta colour which effects a well differentiated visualisation and demarcation from the surrounding tissue enabling analysis of the extent of the newly formed epidermal tissue growing along the wound edges, which we defined as the epithelial tongue (ET). The dimensional state of split formation was also evaluated by this stain.

First, slides were air-dried at room temperature for ten minutes, followed by acetone fixation at -20°C for ten minutes. Afterwards, slides were washed shortly with distilled water, treated with freshly prepared periodic acid and washed again with distilled water. In the next step, slides were covered with Schiff reagent for 15 minutes. To remove unspecific fuchsine all slides were treated in three different cuvettes with freshly prepared sulphurous acid. To increase the colour intensity, slides were washed again under running tap water for ten minutes and then shortly in distilled water. Next, the counter-staining was performed with Meyers haemalum. Slides were dipped into haemalum for 30 seconds liquid and later on washed under running tap water for ten minutes. Finally, slides underwent the alcohol row and xylol solution before embedding like described in chapter 2.2.1. The evaluation of length and area of the ET is illustrated in **Figure 19**. Per section two ETs were evaluated. Using Image J software, a continuous line drawn from the end of BL until the tip of the ET, the length was analysed. The area of the ET was analysed by encircling all newly formed tissue which has grown beyond the former cutting edges.

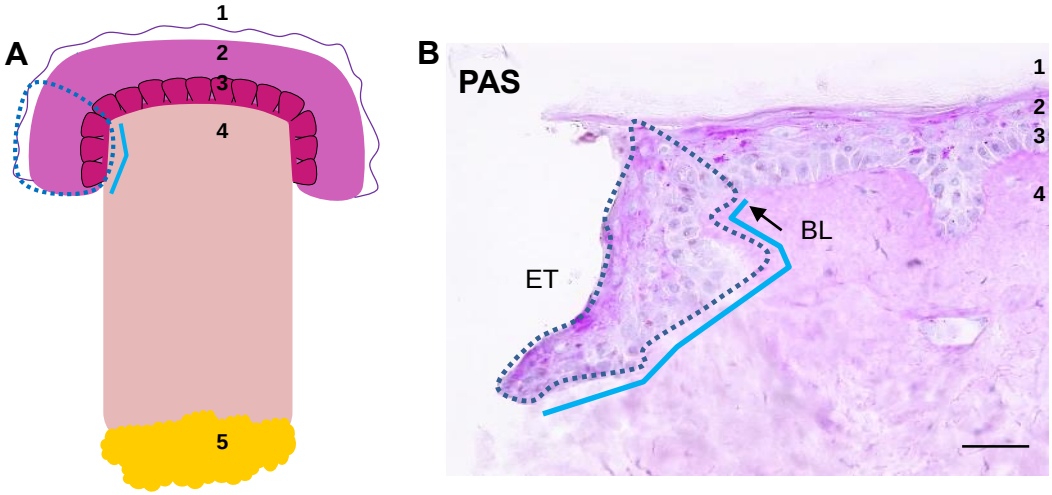

Figure 19 Evaluation of the epithelial tongue

(**A**) Schematic drawing of a punch showing labelled cutaneous layers (1-5). Evaluation of the epithelial tongue (ET) was performed by measuring the marking of the area (dark blue dashed line) and the length (light blue continuous line) which is shown at the left ET in the draft. (**B**) Periodic acid–Schiff stained skin section of the left ET with marked area (dark blue dashed line), length (light blue continuous line), ending of the basal lamina (BL) and cutaneous layers (1-4). 1 = cornified layer, 2 = granular and spinous layer 3 = basal lamina, 4 = dermis, 5 = subcutis. Scale bar: 50 µm.

The extent of subepidermal split formation was evaluated by semi-quantitative determination of the degree of separation along the entire epidermis of PAS stained skin sections. Every image was ordered into 1 of 4 categories after the following scheme: 1 = less than a 1/4th of the epidermis is detached; 2 = more than 1/4th of the epidermis is detached; 3 = more than ½ of the epidermis is not in touch with the dermis and 4 = less than 1/4th of the epidermis is in contact with the dermis. **Figure 20** shows how split formation was analysed in a skin section of “pathological” culture conditions.

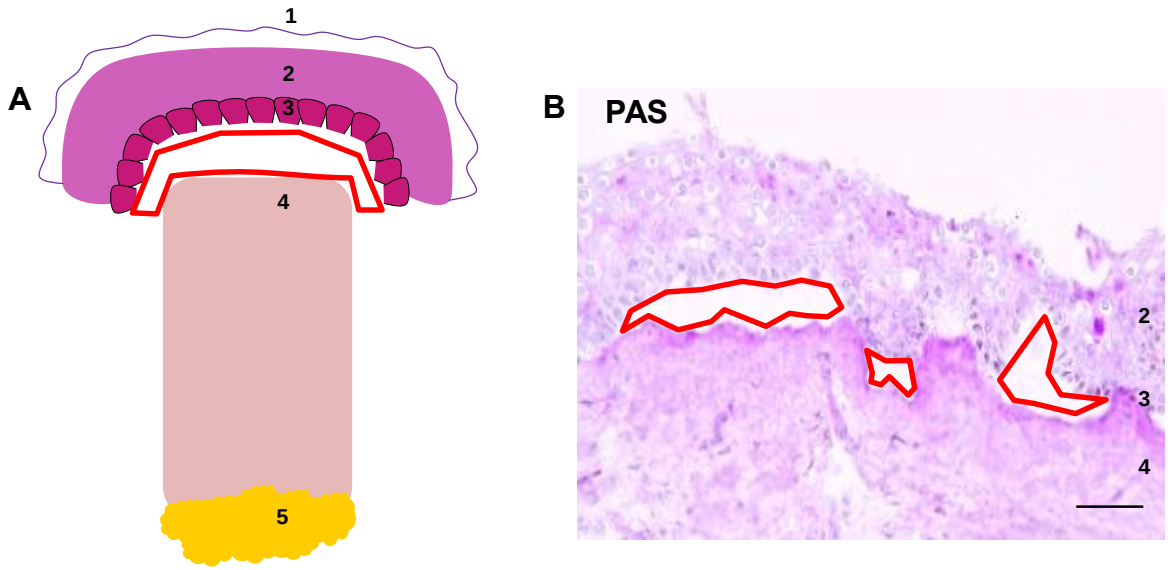

Figure 20 Evaluation of split formation

(**A**) Schematic drawing of a punch showing labelled cutaneous layers (1-5). Assessment of split formation (red continuous line) was performed by a semi quantitative method of rating the gap between epidermis and dermis (red continuous line), with category 1-4. (**B**) Periodic acid–Schiff stained section ("pathological" medium) of the epidermis with split formation (red continuous line) rated at 3 (more than ½ of the epidermis is detached from the dermis) and cutaneous layers (1-4). 1 = cornified layer, 2 = granular and spinous layer, 3 = basal lamina, 4 = dermis, 5 = subcutis. Scale bar: 50 µm.

4.3 Immunofluorescence analyses

Both immunofluorescence (IF) stainings used in this thesis are based on established staining protocols (Bodó et al., 2010; Lu et al., 2007; Meier et al., 2013). Slides for CD31 and CK6 were prepared by fixation in acetone at -20°C, washed three times in Tris-buffered-saline (TBS), pH 7.6. Incubation of the primary antibody was performed over-night at 4°C. For CD31, we used the primary antibody diluted in a ratio of 1:30 in DAKO® antibody diluent and for CK6 the primary antibody was diluted in progen® antibody diluent with a ratio of 1:10. After washing the slides in TBS, the second antibody was applied and incubated again for 45 minutes. It was than conjugated to FITC (fluorescein isothiocyanate) in case of CK6, or conjugated to rhodamine in case of CD31 diluted in JIR Antibody diluent ratio 1:200. After washing the slides in TBS, slides were counter stained by using 0.1 µg/ml 4'6-diamidin-2-phenylindol (DAPI) to highlight the nuclei. Afterwards, slides were washed again in TBS. Sections were embedded in Fluoromount-G® and coated by a coverslip. For each experiment the

primary antibody was omitted on one section to control the unspecific binding of the secondary antibody.

For the assessment of immunoreactivity (IR) all microscopic photographs were taken at the same exposure time and magnification. The intensity of the IF CK6 and CD31 expression signal was normalised by taking the mean of all standard medium values as 100%. The percentages of each test group measured intensity value to the normalized control (100%) were compared. This method was described before (Knuever et al., 2012; Sugawara et al., 2012).

4.3.1 Detection of wounded epithelium using cytokeratin 6 staining

In the first experimental setup, we included the whole epidermis for the measurements of all samples. In the second experimental setup, the area of the ET was analysed. The epidermal parts and the ETs were marked with a freehand drawing function and measured in arbitrary units (**Figure 21**).

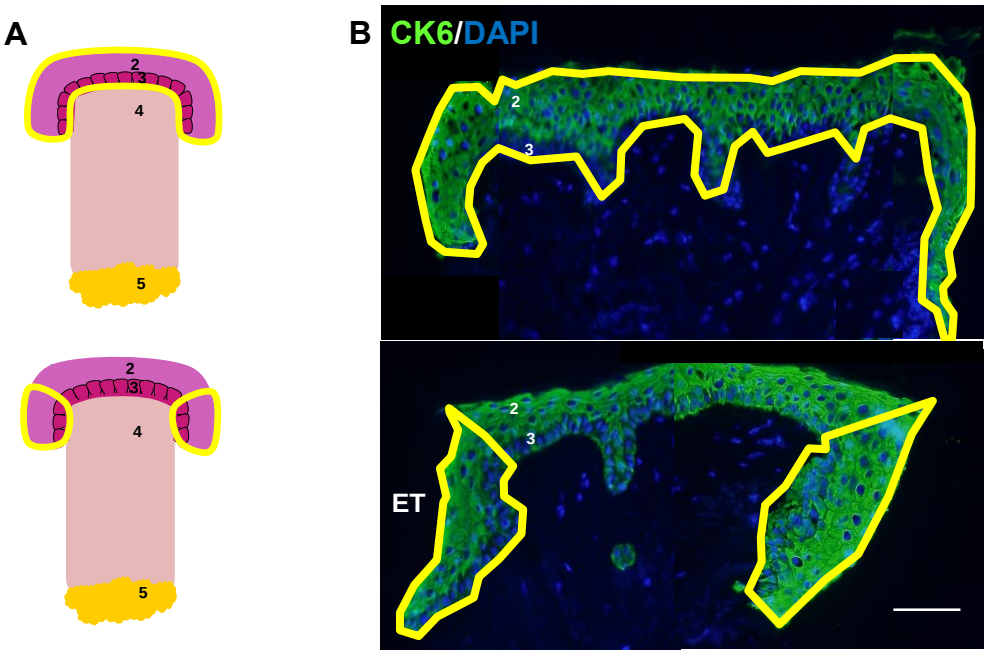

Figure 21 Detection of cytokeratin 6

(**A**) Schematic drawings of the punches show labelled cutaneous layers (1-5). The assessment of cytokeratin 6 (CK6) immunoreactivity (IR) (yellow continuous line) was either performed of the whole epidermis (first experimental setup) or of the epidermal tongues (ETs) (second experimental setup). (**B**) Immunofluorescent CK6/DAPI stainings which were analysed by quantitative immunofluorescence morphometry. 2 = granular and spinous layer, 3 = basal lamina, 4 = dermis, 5 = subcutis; ET: epithelial tongue. Scale bar: 50.0 µm.

Evaluation of angiogenesis using CD31 staining

According to previous assays using CD31 as an appropriate marker for angiogenesis we utilised this method to analyse angiogenesis of wounded skin (Ansell et al., 2011). Stained sections were evaluated by three different categories. To analyse angiogenesis in the various culture conditions the following parameters were investigated:

1. Mean intensity of CD31 IR per visual field.
2. Total number of CD31 positive nuclei per visual field.
3. Total number of CD31 positive lumina per visual field.

All criteria were analysed in three different visual fields in the dermis of an analysed section. Two dermal visual fields were located in immediate vicinity to the newly grown ET on each side of the punch and the third visual field was located in the dermis, middle of the punch part, distance of 200 µm apart from the BL (see **Figure 22**).

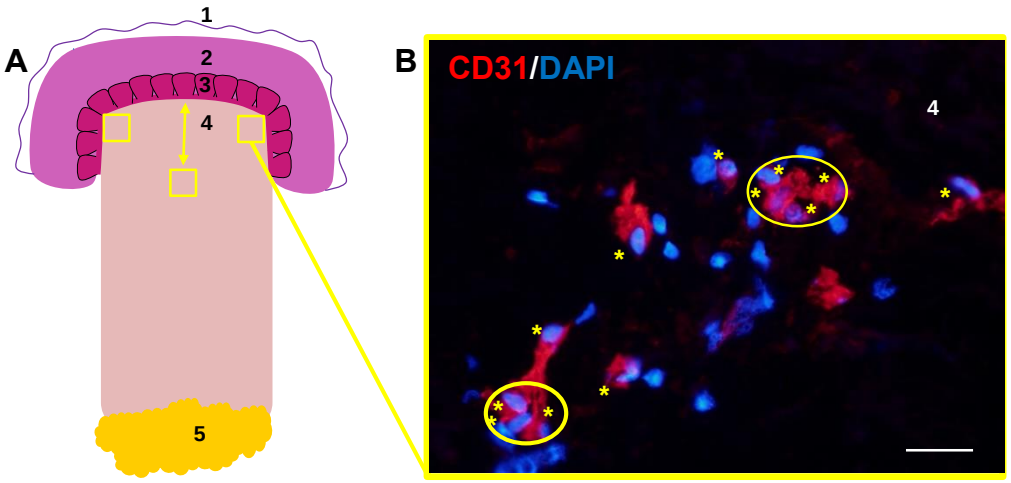

Figure 21 Evaluation of CD31

(**A**) Schematic drawing of a punch showing labelled cutaneous layers (1-5) and three yellow boxes (reference areas) indicating where the visual fields were captured for the evaluation of angiogenesis. (**B**) Immunofluorescent CD31/DAPI staining of a defined dermal area which was analysed by quantitative immunofluorescence morphometry, counting the total number of CD31 positive cells (yellow stars) and vessel lumina (yellow circles) per visual field. 1 = cornified layer, 2 = granular and spinous layer, 3 = basal lamina, 4 = dermis, 5 = subcutis. Scale bar: 50 µm.

4.4 Double-immunofluorescence analyses

The Ki-67/Tdt-mediated dUTP-biotin nick end labelling (TUNEL) double IF was performed for the detection of proliferating (Ki-67) and apoptotic (TUNEL) according to successful established protocols (van Beek et al., 2008; Bodo et al. 2009).

Preparing the slides for the Ki-67/TUNEL stain began with fixation in 1% paraformaldehyde in phosphate buffered saline (PBS), pH 7.6, washing in PBS and post-fixation in glacial acetic acid at -20°. In the next step, the equilibration buffer (70% reaction buffer and 30% terminal desoxynucleotidyl transferase (TdT)-enzyme) was deposited. 13 µl were carefully pipetted on each section and then all slides were incubated at 37°C for 60 minutes. Subsequently a stop buffer which was warmed up to 37°C and mixed with *aqua dest.* was put on each section. All slides were washed in PBS and were later pre-incubated with goat serum (10% in PBS) for 20 minutes. Incubation of the primary antibody (mouse-anti human Ki-67 Antigen, 1:20 in PBS, 2% goat serum) was performed overnight at 4°C. After washing the slides in PBS, the fluorescence staining was performed by the TUNEL-Kit, anti-digoxigenin antibody which was incubated for 30 minutes. Slides were washed in PBS again and the second fluorescence staining was applied by goat anti-mouse-IgG-rhodamine red (1:20 in PBS + 2% goat serum).

After washing the slides in PBS, slides were counter stained by using 0.1 µg/ml DAPI to detect nuclei. Afterwards, slides were washed again in PBS. Sections were embedded in Fluoromount-G® and covered by a coverslip. For each experiment the primary antibody was omitted on one section to control the unspecific binding of the secondary antibody.

4.4.1 Proliferation and apoptosis evaluation using Ki-67/TUNEL staining

To quantify proliferating and apoptotic cells of the epidermis and the ET, Ki-67-, TUNEL-, and DAPI positive cells were counted. In the first part of the thesis, cells were counted inside a reference area covering the ETs and two parts of the epidermis inside the punch (**Figure 23**). In the second experimental setup, the evaluation was focused on the newly formed epidermal tissue at the wound edges, the ET which is an established method (Ramot et al., 2011) .The amount of Ki-67 positive and TUNEL positive cells was calculated as a percentage of all DAPI positive cells as described before (Gáspár et al., 2011; Holub et al., 2012).

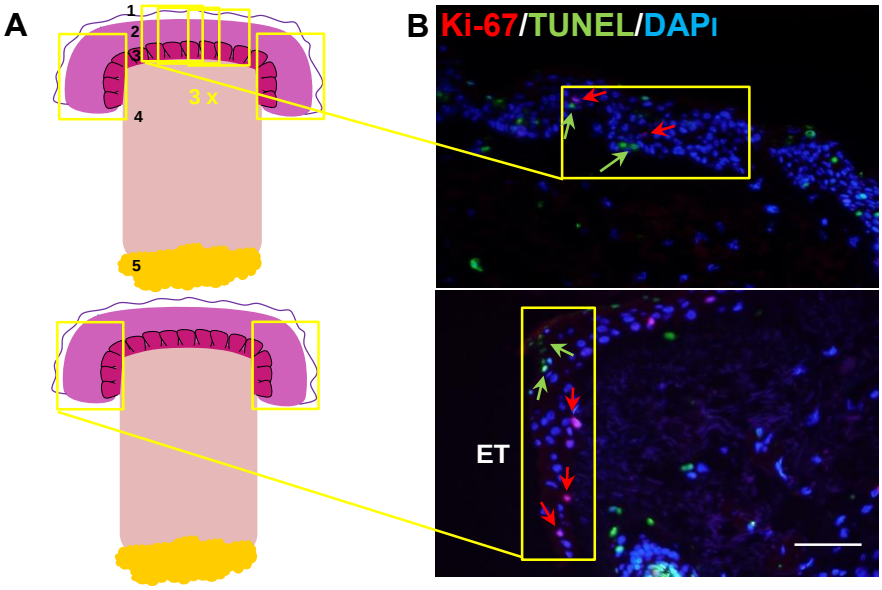

Figure 22 Evaluation of Ki-67/TUNEL

(**A**) Schematic drawings of the punches showing labelled cutaneous layers (1-5) and reference areas (yellow boxes) above the epithelial tongues (ETs) and 3 more epidermal areas inside the punch (first experimental setup) and in the draft below, reference areas are placed above the ETs (second experimental setup). (**B**) Double immunofluorescent Ki-67/TUNEL/DAPI staining of the epidermis and left ET with reference areas (yellow boxes). Red fluorescent immunoreactivity (IR) represents Ki-67 positive (proliferating, red arrows), green flurorescent IR represents TUNEL positive (apoptotic, green arrows) and blue fluorescent IR represents DAPI positive (all nuclei). 1 = cornified layer, 2 = granular and spinous layer, 3 = basal lamina, 4 = dermis, 5 = subcutis; ET: epithelial tongue. Scale bar: 50.0 µm.

4.5 Microscope

For the evaluations of the skin sections, microscopic photographs were captured with a Biozero-8000 microscope. Magnifications alter depending on the parameters which needed to be analysed. This microscope supports brightfield and fluorescence observation. Hence, all images were taken with the same instrument and settings were saved to standardise the conditions for all analyses.

4.6 Software

All histochemical and IF stained images were analysed by using image J software. This program is an open source Java image processing program (https://imagej.net/ImageJ, 10.08.2017). For each evaluation a calibrated scale bar was first set and then applied on every image with the same dimension. All images were orientated on a 50 µm scale bar except CD31 stained images which were orientated on a 200 µm scale bar. The length and area of the ETs was measured in µm and μm^2, respectively. The determination of the numbers of certain cell type was performed with the "cell-counter" plugin (dyskeratosis, Ki-67/TUNEL, CD31). The intensity measurements of the fluorescencent stainings CD31 and CK6 were run after calibration and the "mean-intensity" was recorded.

4.7 Statistical analyses

Unpaired t-test and Mann-Whitney-U-test were applied, depending on the Gaussian distribution. All data was expressed as mean ± SEM (standard error of the mean); p-values *<0.05, **≤0,01, ***≤0,001 were regarded as significant which was considered by using the statistical analysis software Prism v. 3.00 (Graph-Pad Software Chicago, IL, USA). For every parameter, two punches were cultured and compared. Skin tissue of six different female donors were used to run all experiments, skin of HS 12-106 – HS 12-147 for the first experimental setup and skin of HS 12-150 – HS 13-090 for the second experimental setup (see Table 2) meaning three different patients per experimental setup. The resulting data was pooled since the resulting data trended to be highly comparable which resulted in a total of 19–54 skin fragments per experimental group.

5 Results

5.1 Three-dimensional organ culture full-thickness wound healing model can be transformed to a “pathological” wound healing model

In order to establish a “pathological” short-term WH model with human skin, organ culture conditions were adjusted by imitating the setting in which chronic ulcers develop. To fulfil the requirements for our “pathological” WH model which were to be quickly inducible, easily reproducible and close to clinical reality, we modified the culture medium as follows: (i) withdrawal of insulin, (ii) adding a high concentration of glucose, (iii) inducing oxidative stress and (iv) cultured skin punches under hypoxic conditions. All punches were compared after 3 days of culture duration. The cells and ECM of the skin punches which were exposed to such high metabolically stress for 3 days, were in a large part damaged but still revealed signs of tissue viability. To quantify the extend of this tissue damage, we first established two new histomorphometric read-out parameters: (i) counting dyskeratotic KCs and (ii) analysing the extend of subepidermal split formation between epidermis and dermis. The results of these newly developed methods were in row with the analyses of the proven histomorphometric methods to quantify reepithelialisation, proliferation/apoptosis, CK6 expression and angiogenesis (Meier et al., 2013; med. diss., Zhang, 2013).

5.1.1 “Pathological” culture conditions induced major changes in epidermal morphology

We hypothesized that “pathological” WH conditions would negatively influence skin structure and integrity, even in short-term organ culture. In order to ascertain the impact of “pathological” WH conditions upon KC differentiation and skin integrity, when compared to standard full-thickness skin organ culture, 4 mm skin punches were cultured in standard or modified culture media (hyperglycaemic, hypoinsulinaemic and in the presence of H_20_2 under hypoxic conditions). It is shown that indeed, dyskeratosis (**Figure 24**) and subepidermal separation (split formation) (**Figure 25**) were both significantly increased in the “pathological” conditions. Split formation and dyskeratosis are two characteristic criteria of skin, cultured under “pathological” conditions.

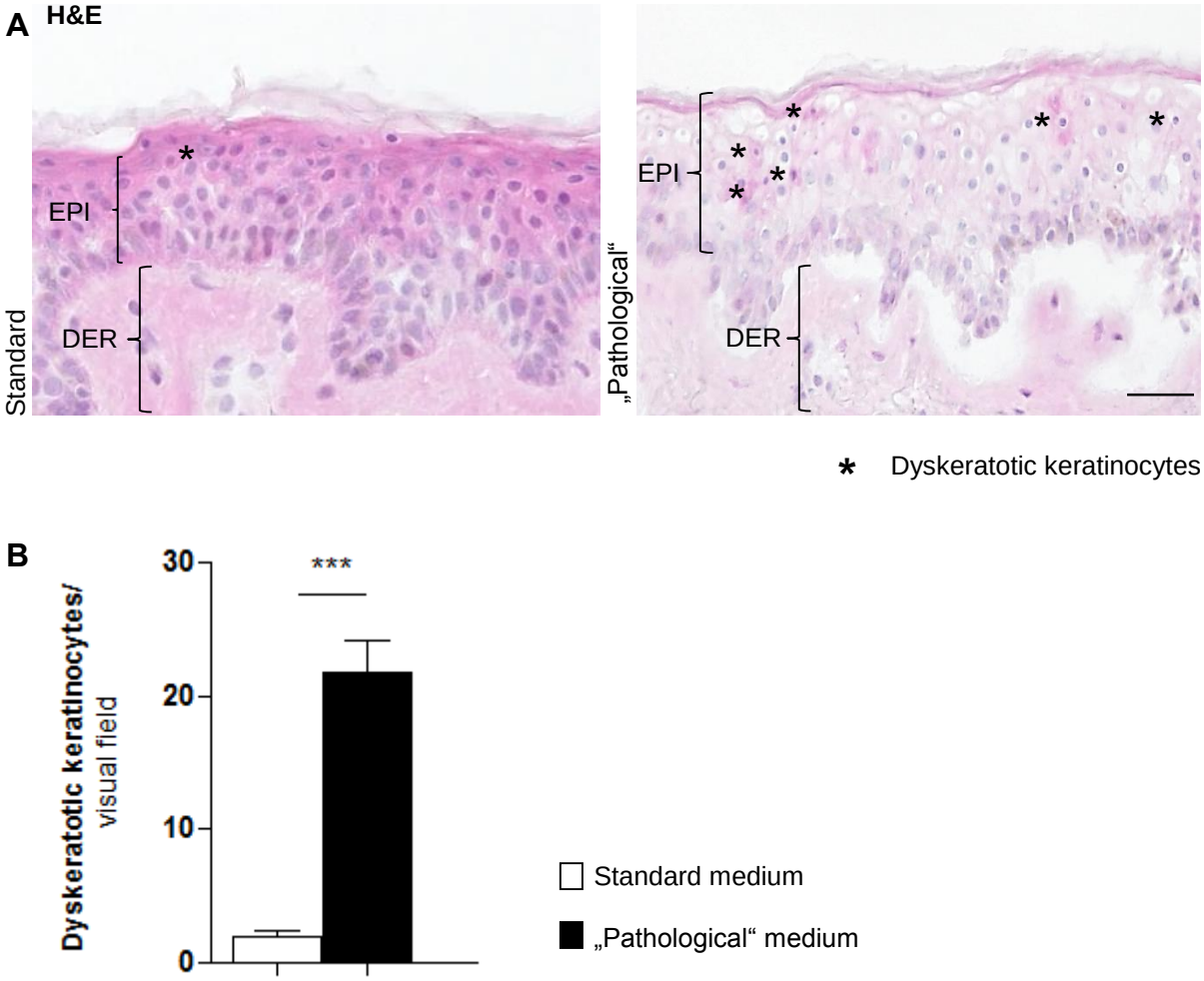

Figure 23 “Pathological” medium conditions induce dyskeratosis

(**A**) Haematoxylin and eosin staining of skin punches from standard and “pathological” culture conditions showing the epidermis and labelled dyskeratotic keratinocytes (KC)s (black stars).

(**B**) Dyskeratotic KCs were counted inside the whole epidermis per visual field. Dyskeratosis was a predominant phenomenon under “pathological” culture conditions.

EPI: epidermis; DER: dermis; scale bar: 50µm; ***p≤0.001; pooled data from 3 different patients, 5-6 punches, 23-44 skin sections; mean ± SEM; p-value was calculated by Mann-Whitney-U test for unpaired samples.

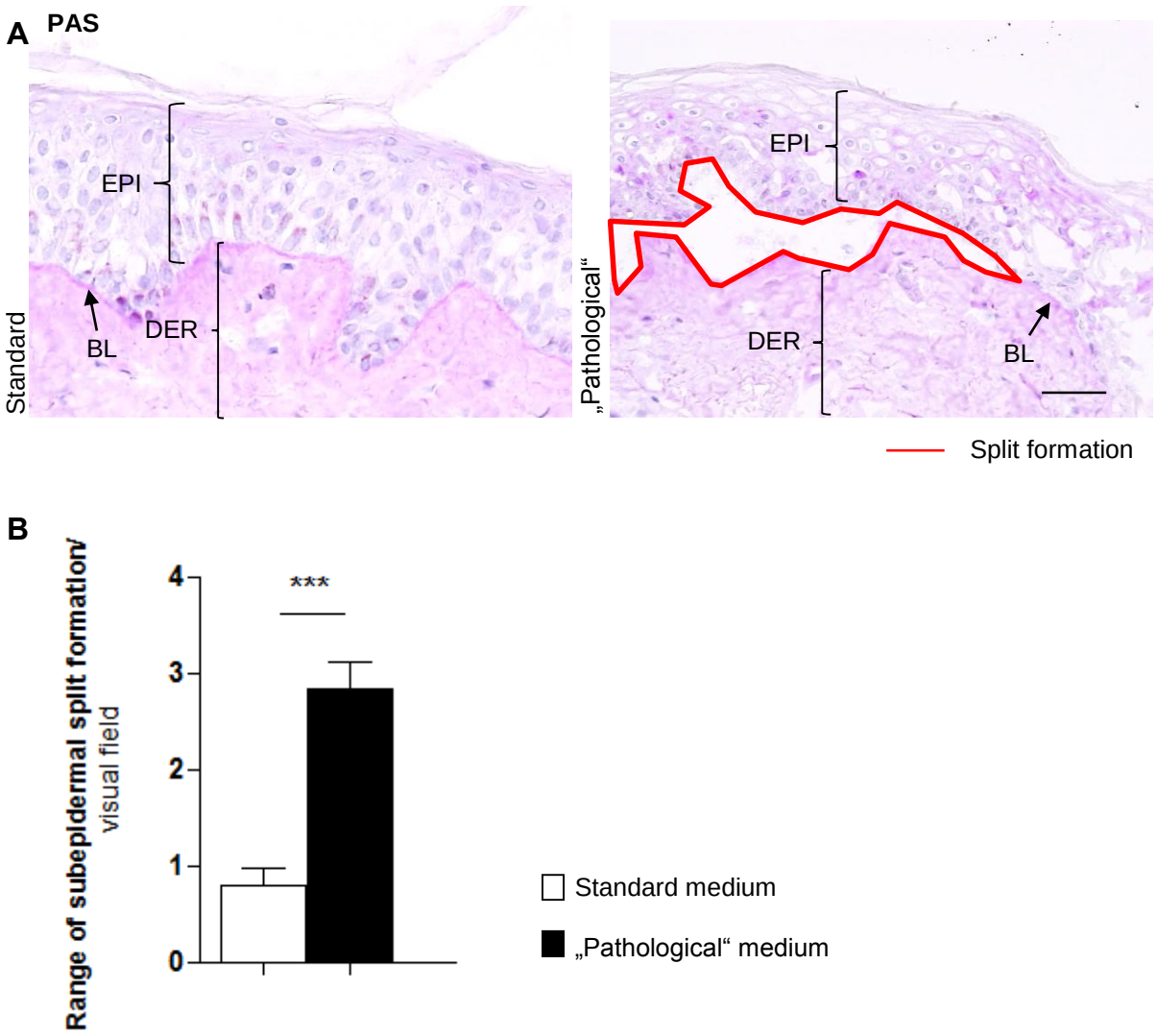

Figure 24 “Pathological” medium conditions induce a detachment of the epidermis from the dermis (= split formation)

(**A**) Periodic acid-Schiff stained sections represent the different manifestation of split formation (red continuous line) comparing standard and “pathological” cultured skin.

(**B**) The extend of split formation was assessed by semi-quantitative method per visual field and it was significantly higher detectable in skin from “pathological” culture conditions

EPI: epidermis; DER: dermis; BL: basal lamina; scale bar: 50 µm; ***p≤0.001; pooled data from 3 different patients, 6 punches, 38-44 skin sections; mean ± SEM; p-value was calculated by Mann-Whitney-U test for unpaired samples.

5.1.2 Epithelial tongue formation is reduced under “pathological” conditions

KCs migrate from the BL along the wound edge to cover the tissue defect during the process of reepithelialisation (Eming and Tomic-Canic, 2017; Pastar et al., 2014). For analysing the rate of reepithelialisation we assessed the length and area of the ET, which has developed 3 days after the punch biopsy (= injury) by those migrating KCs. Wounded human skin punches which were impaired by the “pathological” conditions (hyperglycaemia, withdrawal of insulin, hypoxia and oxidative stress) were characterised by a rarely developed ET (**Figure 26**). This evaluation indicates that “pathological” conditions harm reepithelialisation and also correlate with the morphological alterations of a higher incidence of dyskeratotic KCs and loss of epidermal layer integrity, evidenced by a higher range of epidermal split formation under “pathological” conditions.

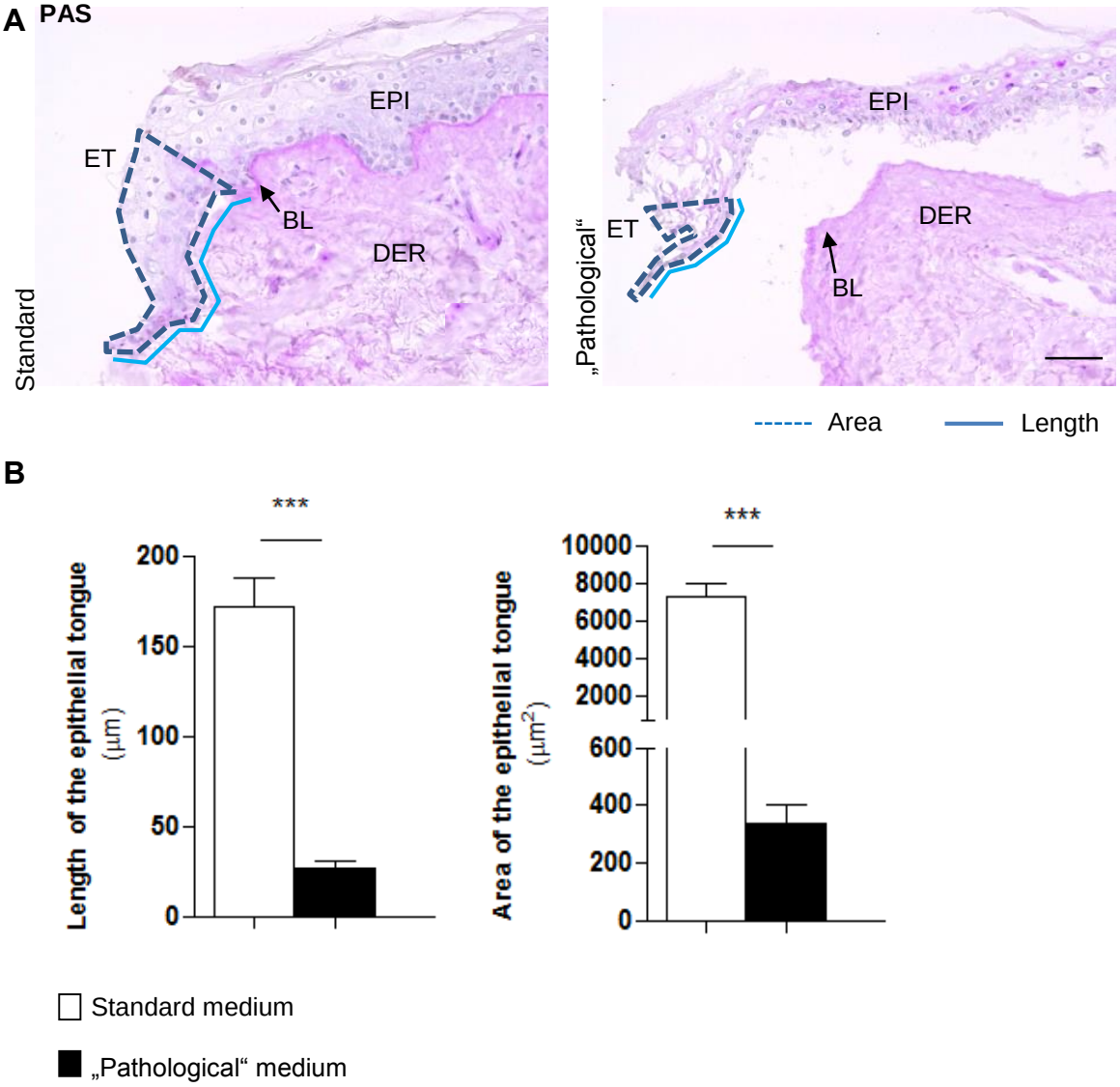

Figure 25 Reepithelialisation is low under “pathological” medium conditions

(**A**) Periodic acid-Schiff staining of the left wound edges with a physiologically developed epithelial tongue (ET) under standard conditions and a little developed ET under “pathological” conditions. Evaluations were performed by measuring the marking of the length (light blue continuous line) and the area (dark blue dashed line).

(**B**) Comparing the sizes (length and area) of the ETs from standard and “pathological” conditions there are highly significant results of a reduced ET size under “pathological” conditions.

ET: epithelial tongue; EPI: epidermis; DER: dermis; BL: basal lamina; scale bar: 50µm; ***p≤0.001; pooled data from 3 different patients, 6 punches, 26-29 skin sections; mean ± SEM; p-value was calculated by Mann-Whitney-U test for unpaired samples.

“Pathological” medium conditions induce increased cell death and decreased proliferation of keratinocytes

To further investigate the features of KCs under “pathological” medium conditions during reparative processes, percentages of Ki-67 positive (proliferative) and TUNEL positive (apoptotic) KCs were assessed. Proliferative KCs were significantly reduced in the epidermis of “pathological” cultured skin tissue. Conversely, apoptotic KCs were found in a high rate inside the epidermis of skin cultured under “pathological” conditions (**Figure 27**). It is also remarkable that the interval of the percentages between proliferative and apoptotic KCs is very small which indicates a balanced relation of KCs undergoing an appropriate life cycle and is in contrast to the ratio of proliferative and apoptotic KCs under “pathological” conditions with a very high interval in-between. With this, we proved the establishment of “pathological” medium conditions once more because there is a severely reduced cell viability but still a small number of proliferative cells detectable.

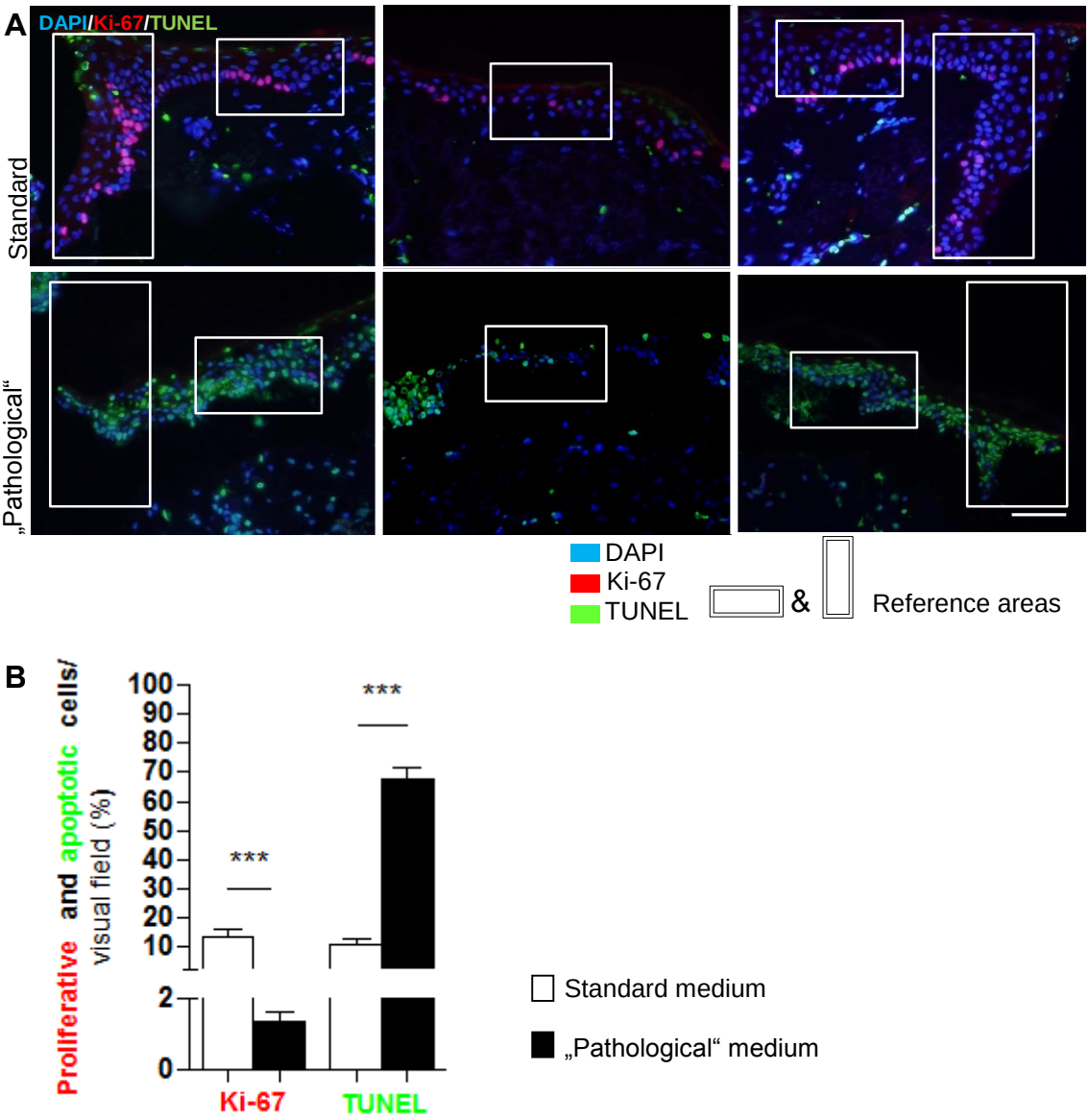

Figure 26 “Pathological” medium conditions rearrange the balance of Ki-67 and TUNEL positive cells (**A**) Double immunofluorescent Ki-67/TUNEL/DAPI staining of left and right epithelial tongues (ETs) and the epidrmis. Red fluorescent immunoreactivity (IR) represents Ki-67 positive (proliferating), green flurorescent IR represents TUNEL positive (apoptotic) and blue fluorescent IR represents DAPI positive (all nuclei) which were counted separately in defined reference areas (white boxes).

(**B**) The amounts of proliferative and apoptotic cells are provided as percentages of all cells (number of DAPI positive cells was set as 100%) inside the reference area from the ETs and epidermis. The number of proliferative cells is significantly lower and apoptosis is highly increased under “pathological” conditions.

Scale bar: 50 µm; ***p≤0.001; pooled data from 3 different patients, 6 punches, 31-35 skin sections; mean ± SEM; p-value was calculated by Mann-Whitney-U test for unpaired samples.

5.1.3 Cytokeratin 6 expression is decreased under “pathological” wound healing conditions

CK6 is an essential keratin of the epidermis (Windoffer et al., 2011). It plays a major role in WH and its expression is upregulated in company of stressful stimuli (Rotty and Coulombe, 2012).

In **Figure 28** it is shown that we analysed the CK6 IR of the whole epidermis which was clearly lower under “pathological” medium conditions. IFs demonstrate an incoherent net of the whole epidermal structure resulting in less cellular capacity for even expressing CK6. Evaluations show a significant decrease of the WH associated CK6 in the whole epidermis of skin from “pathological” WH conditions.

In summary, it can be noticed that “pathological” circumstances impede the adequate CK6 production which usually is an important response upon skin injury (Wojcik et al., 2000).

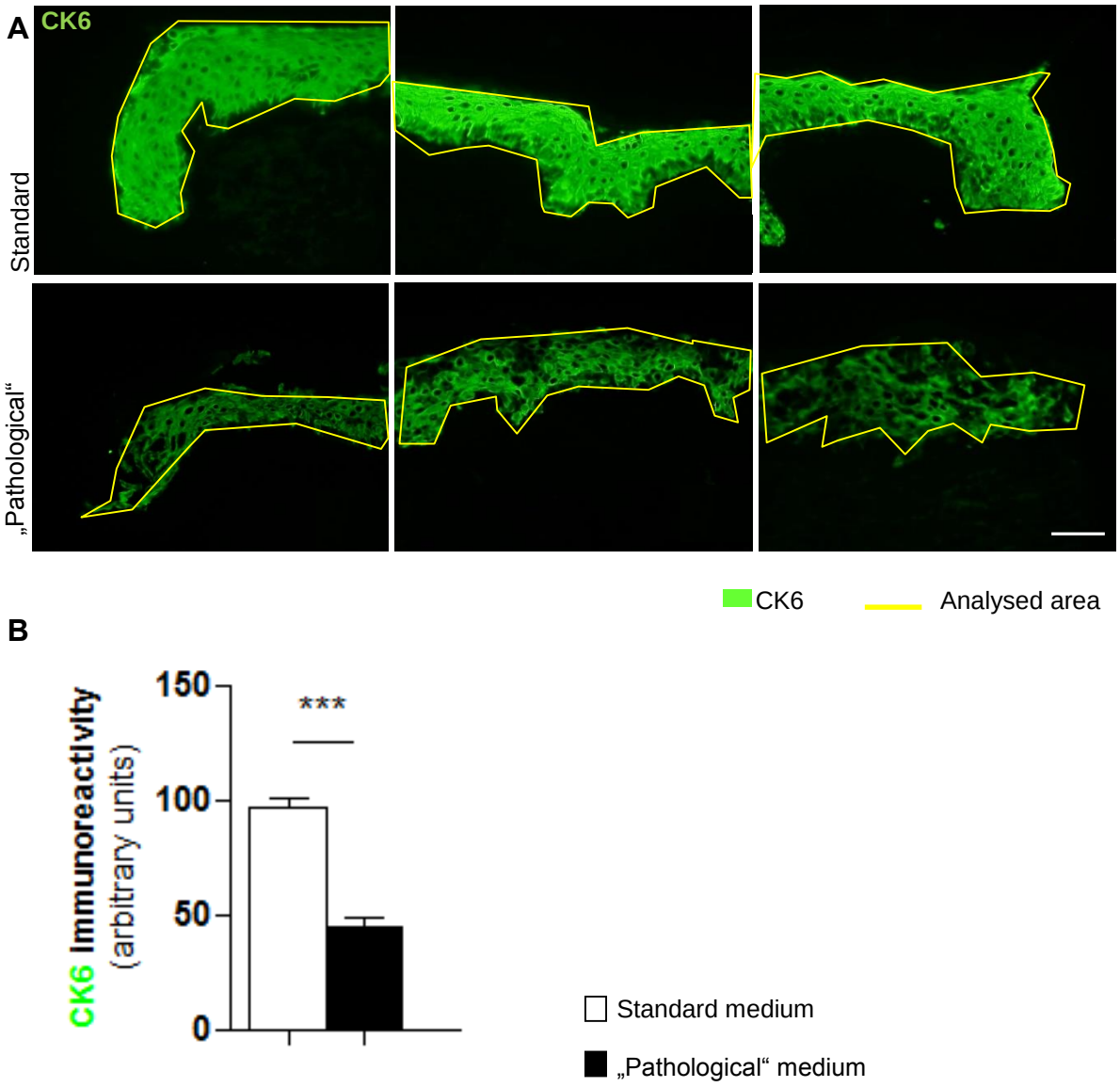

Figure 27 Cytokeratin 6 immunoreactivity is impaired under “pathological” medium conditions

(**A**) Immunofluorescent cytokeratin 6 (CK6) staining of skin sections showing left and right epithelial tongues (ETs) and the epidermis Green fluorescent immunoreactivity (IR) represents the CK6 positive signal and appears much brighter in the skin from standard medium conditions. The CK6 positive IR was assessed in the epidermis (yellow continuous line) by quantitative immunofluorescence morphometry.

(**B**) CK6 protein expression in the “pathological” cultured skin was significantly reduced compared to normalised results of the standard medium (=100%).

Scale bar: 50 µm; ***p≤0.001; pooled data from 3 different patients, 6 punches, 34-35 skin sections; mean ± SEM; p-value was calculated by Mann-Whitney-U test for unpaired samples.

5.1.4 Intracutaneous angiogenesis is strongly impaired by metabolically negative treats

According to our findings that WH processes of the induced “pathological” WH model are not functioning fundamentally, we found that there is little angiogenesis occurring in the “pathological” sections. This statement is based on our findings provided by CD31/DAPI staining, which was quantified by the three categories: (i) mean intensity of CD31 IR, (ii) total number of CD31 positive nuclei and (iii) total number of lumina. Dermal reference areas of the skin from “pathological” conditions show a reduced CD31 IR and very low amount of CD31 positive cells (= endothelial cells) and lumina compared to the dermal parts of the skin cultured under standard conditions (**Figure 29**).

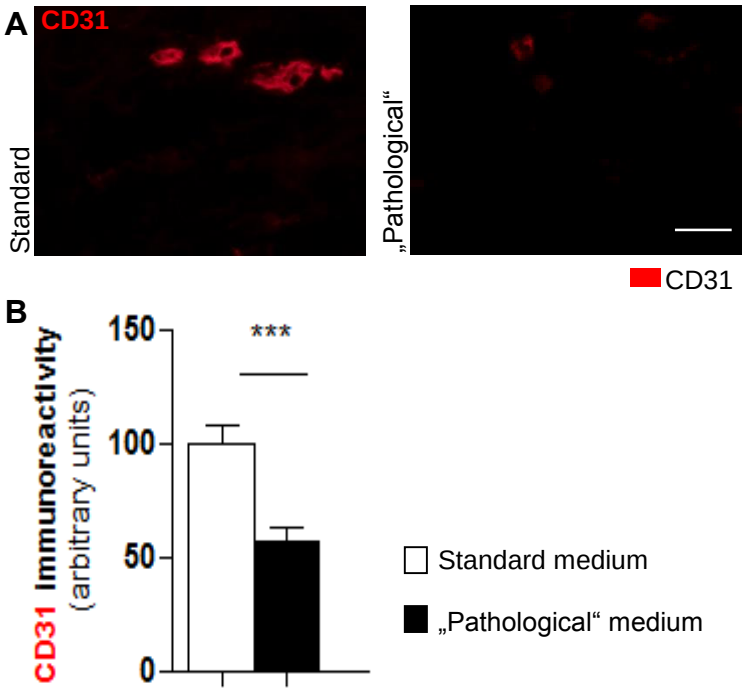

Figure 28 CD31 immunoreactivity is reduced under “pathological” medium conditions

(**A**) Immunofluorescent CD31 staining of an analysed part of the dermis demonstrating a clear red fluorescent CD31 positive signal whilst this is hardly detectable in the skin of “pathological” medium conditions.

(**B**) The immunoreactivity (IR) was assessed per visual field of three defined dermal reference areas comparing “pathological” medium conditions to normalised results of the standard medium conditions (=100%). CD31 IR was significantly less detected in the “pathological” sections.

Scale bar: 50 µm; ***p≤0.001; pooled data from 3 different patients, 6 punches, 41-42 skin sections; mean ± SEM; p-value was calculated by Mann-Whitney-U test for unpaired samples.

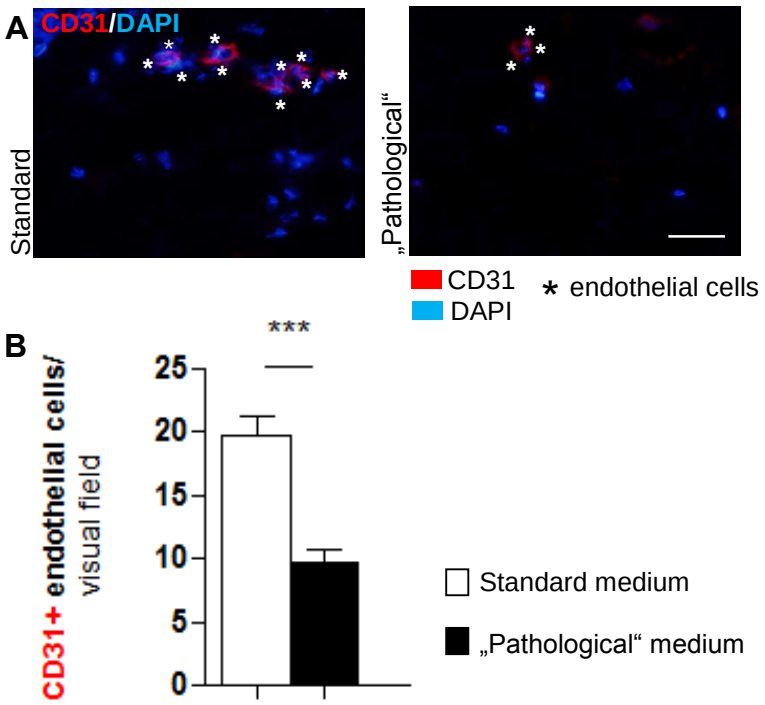

Figure 29 CD31 positive endothelial cells are reduced under “pathological” medium conditions

(**A**) Immunofluorescent CD31/DAPI staining of a defined dermal area. An endothelial cell was counted for each blue fluorescent DAPI positive nucleus associated to a red fluorescent CD31 positive signal (white stars) which were sparsely found under “pathological” medium conditions.

(**B**) The number of endothelial cells was assessed per visual field of three defined dermal reference areas comparing the results of “pathological” medium conditions to the results of standard medium conditions. The number of endothelial cells was significantly lower under “pathological” conditions.

Scale bar: 50 µm; ***p≤0.001; pooled data from 3 different patients, 6 punches, 41-42 skin sections; mean ± SEM; p-value was calculated by Mann-Whitney-U test for unpaired samples.

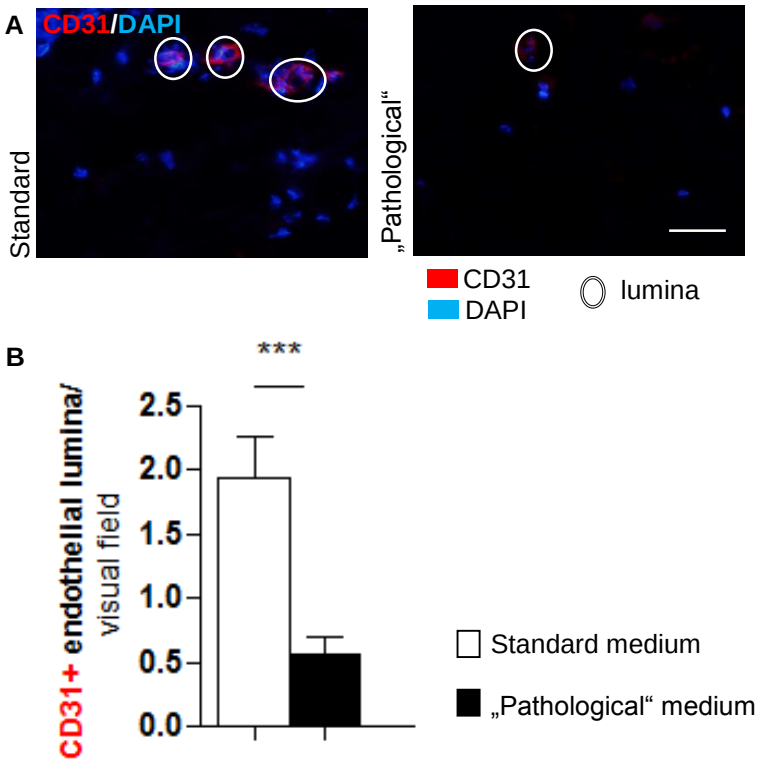

Figure 30 CD31 positive endothelial vessel lumina are reduced under “pathological” medium conditions (**A**) Immunofluorescent CD31/DAPI staining of a defined dermal area. An endothelial lumen (white encircled) was counted for blue fluorescent DAPI positive nuclei associated to a red fluorescent CD31 positive signal forming a lumen.

(**B**) The number of lumina was assessed per visual field of three defined dermal reference areas comparing the results of “pathological” medium conditions to the results of standard medium conditions. The number of lumina was significantly lower under “pathological” conditions.

Scale bar: 50 µm; ***p<0.001; pooled data from 3 different patients, 6 punches, 41-42 skin sections; mean ± SEM; p-value was calculated by Mann-Whitney-U test for unpaired samples.

5.1.5 Thyroxine supplemented medium conditions enhance reepithelialisation

The stimulation of epithelial proliferation by T3 was described before in other tissue culture studies (Safer, 2013) and since it has been shown before that T4 stimulated wound closure in mice (Safer et al., 2005) and in “healthy” organ cultured human skin (med. diss., Zhang, 2013), we wondered if reepithelialisation would also be enhanced by the supplementation of T4 even under “pathological” conditions. After proofing a reduced ET formation under “pathological” conditions by measuring length and area of the newly grown KCs at the wound edges in PAS stainings, we used the same method to define reepithelialisation. The ET formation of skin from T4 supplemented standard medium conditions was compared with the ET formation of standard conditions and the ET formation under T4 supplemented “pathological” medium conditions with “pathological” conditions. In the skin of T4 supplemented standard and “pathological” medium, an increased growth of the ET in terms of length and area could be analysed which might proof that T4 stimulates KC migration (**Figure 32**).

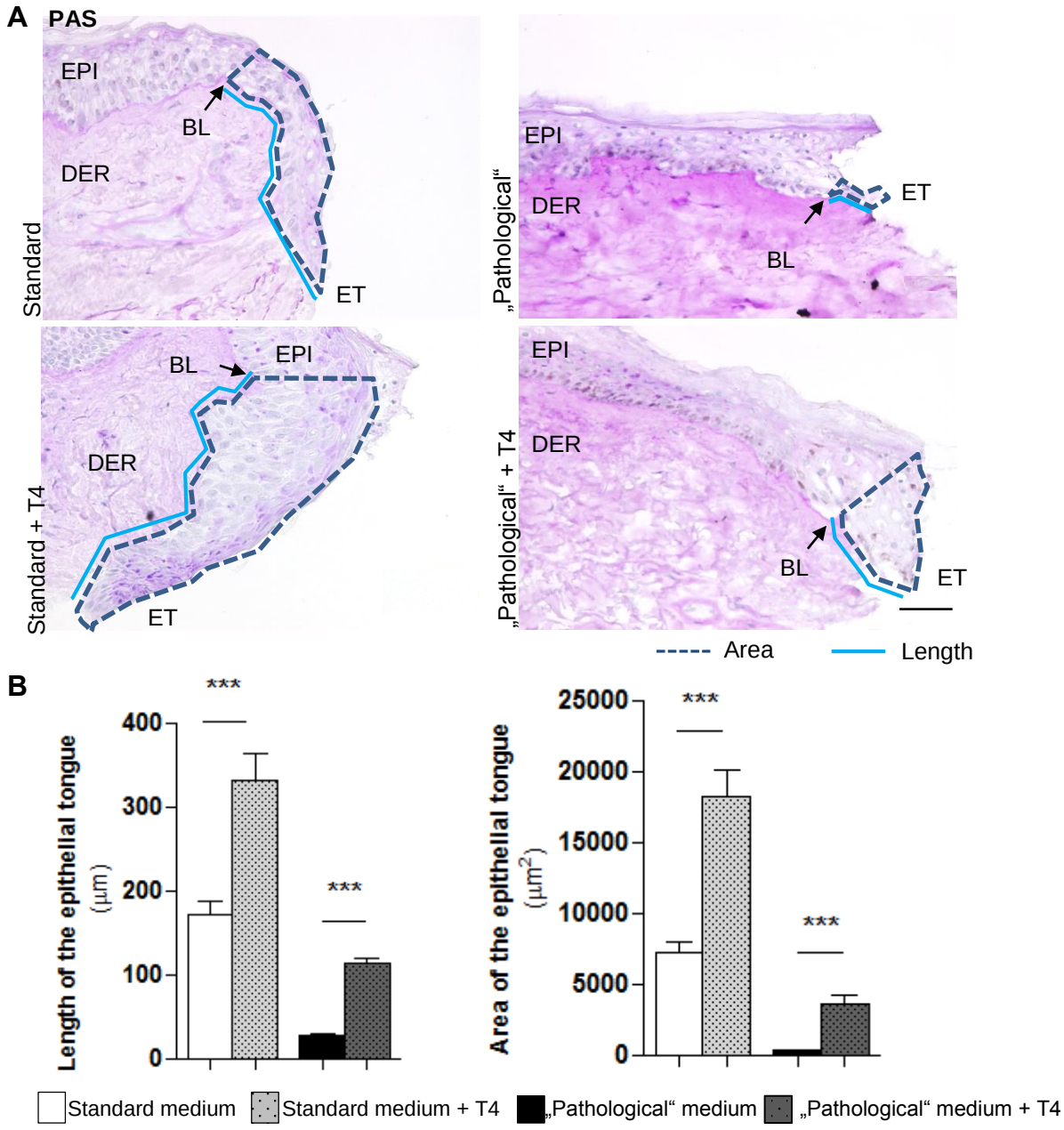

Figure 31 Epithelial tongue formation is enhanced by thyroxine supplementation

(**A**) Periodic acid-Schiff staining of right wound edges and the newly formed epithelial tongue (ET) under standard, standard + thyroxine (T4), “pathological” and “pathological” + T4 medium conditions. Evaluations were performed by measuring the marking of the length (light blue continuous line) and the area (dark blue dashed line) of the ETs.

(**B**) Statistical analyses demonstrate the evaluation of the measured length and area of the ETs comparing standard versus (vs.) standard + T4 conditions and “pathological” vs. “pathological” + T4 conditions. The ETs of the skin cultured in T4 supplemented medium were significantly larger.

ET: epithelial tongue; EPI: epidermis; DER: dermis; BL: basal lamina; scale bar: 50 µm; ***p≤0.001; pooled data from 3 different patients, 6 punches, 24-29 skin sections; mean ± SEM; p-value was calculated by Mann-Whitney-U test for unpaired samples.

5.1.6 Thyroxine supplemented medium conditions cause an increase of proliferative cells under “pathological” medium conditions and a decrease of apoptosis

To further investigate the features of KCs, the influence of T4 medium supplementation was analysed by assessing percentages of Ki-67 positive (proliferative) and TUNEL positive (apoptotic) cells. T4 supplemented standard medium conditions were compared with standard conditions and the T4 supplemented “pathological” medium conditions were compared with “pathological” conditions.

In this second experimental setup, we focused on analysing the ETs (**Figure 33 A**). The ETs of skin punches which were cultured under “pathological” conditions but treated by T4 contained three times less apoptotic cells than the ETs of “pathological” cultured skin punches. There was also a significant decrease of apoptotic cells in the ETs of skin cultured in the T4 supplemented tissue in comparison to the ETs of standard medium conditions (**Figure 33 B**). In terms of proliferation the results were rather severe but showing a significantly positive result concerning the T4 treatment of the “pathological” culture conditions in contrast to the non-treated “pathological” cultured skin tissue, for the tissue remodelling events inside the ET.

These results can confirm the data of the impaired ET formation under “pathological” conditions and enhanced size of the tongue by T4 treatment.

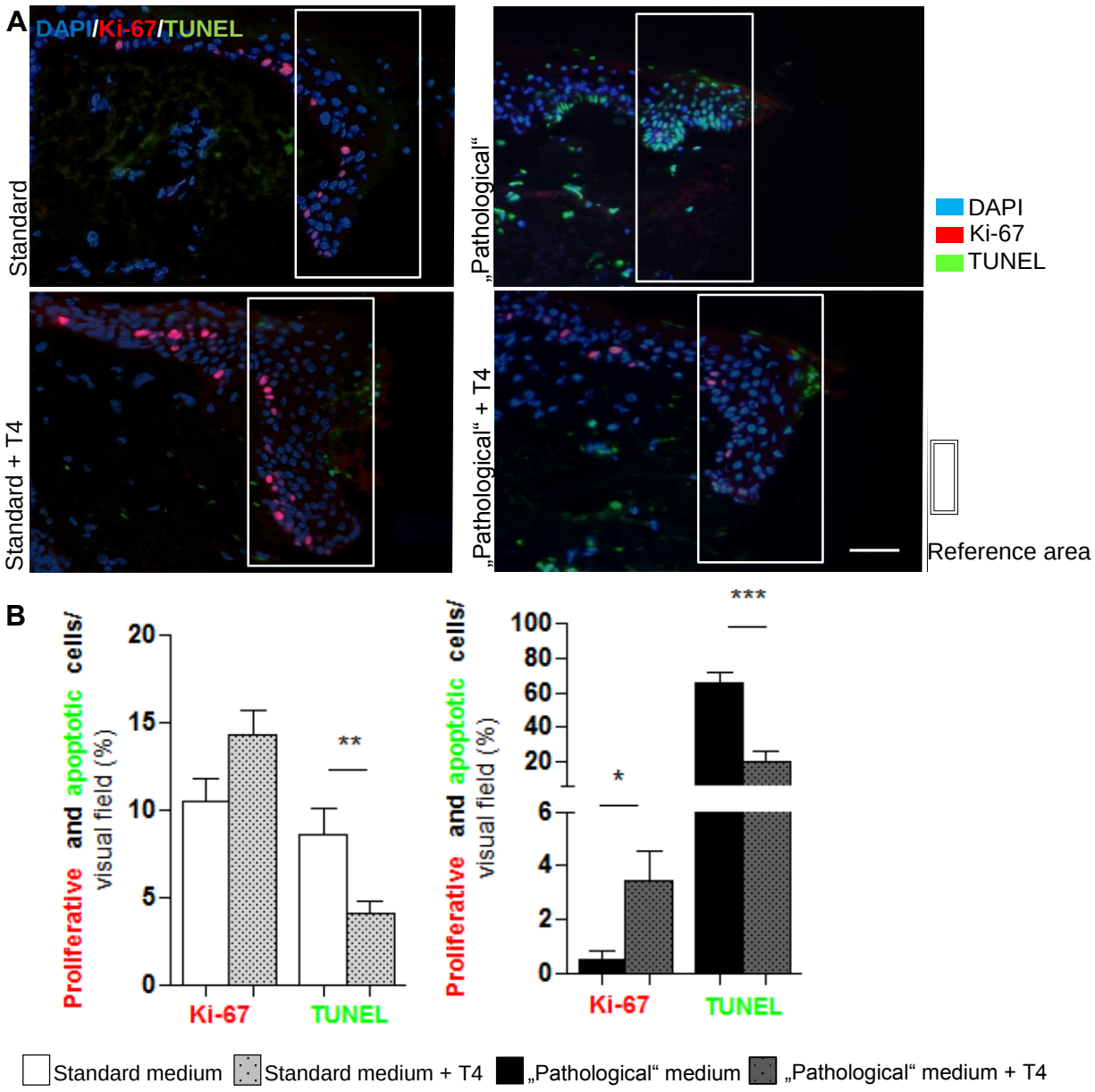

Figure 32 Thyroxine improves the balance of Ki-67- and TUNEL positive cells

(**A**) Double immunofluorescent Ki-67/TUNEL/DAPI staining showing right wound edges and epithelial tongues (ETs). Red fluorescent immunoreactivity (IR) represents Ki-67 positive (proliferating), green flurorescent IR represent TUNEL positive (apoptotic) and blue fluorescent IR represent DAPI positive (all nuclei) which were counted separately in defined reference areas (white boxes). (**B**) Statistical analyses of the amount of proliferative/apoptotic cells comparing standard versus (vs.) standard + thyroxine (T4) conditions and “pathological” vs. “pathological” + T4 conditions. Results of proliferative and apoptotic cells are provided as percentage of all cells (number of DAPI positive cells was set as 100%) inside the reference area from the ETs and epidermis. The amount of proliferative cells trended to be increased under standard + T4 medium conditions but was only significant inside the ETs of “pathological” + T4 medium conditions. Apoptosis was reduced under T4 treated medium conditions most significantly under “pathological” medium conditions. Scale bar: 50 µm; ***p≤0.001, **p≤0.01, *p<0.05; pooled data from 3 different patients, 6 punches, 24-35 skin sections; mean ± SEM; p-value was calculated by Mann-Whitney-U test for unpaired samples.

5.1.7 Cytokeratin 6 expression is increased by the influence of thyroxine under "pathological" conditions

Since the expression of the WH associated keratin 6 is decreased in skin tissue cultured under "pathological" WH conditions and it can be found in literature that there is a TRE located on the promoter region of the skin and T3 is known to increase CK6 expression in murine HFs *in vivo* (Safer et al., 2005), we wanted to investigate if T4 influences the CK6 expression. The evaluation of the T4 treated skin was performed in the ETs (**Figure 34 A**).

In the T4 supplemented standard medium conditions, CK6 expression was not significantly increased compared to standard medium conditions but in the T4 treated "pathological" medium group there was a significantly higher CK6 IR detectable than in the untreated "pathological" conditions. Even though it is described that TH can influence CK6 expression we can only find a significant difference of CK6 IR under "pathological" culture conditions. These findings provide the first suggestion of a sort of cell protective effect of T4 (**Figure 34 B**).

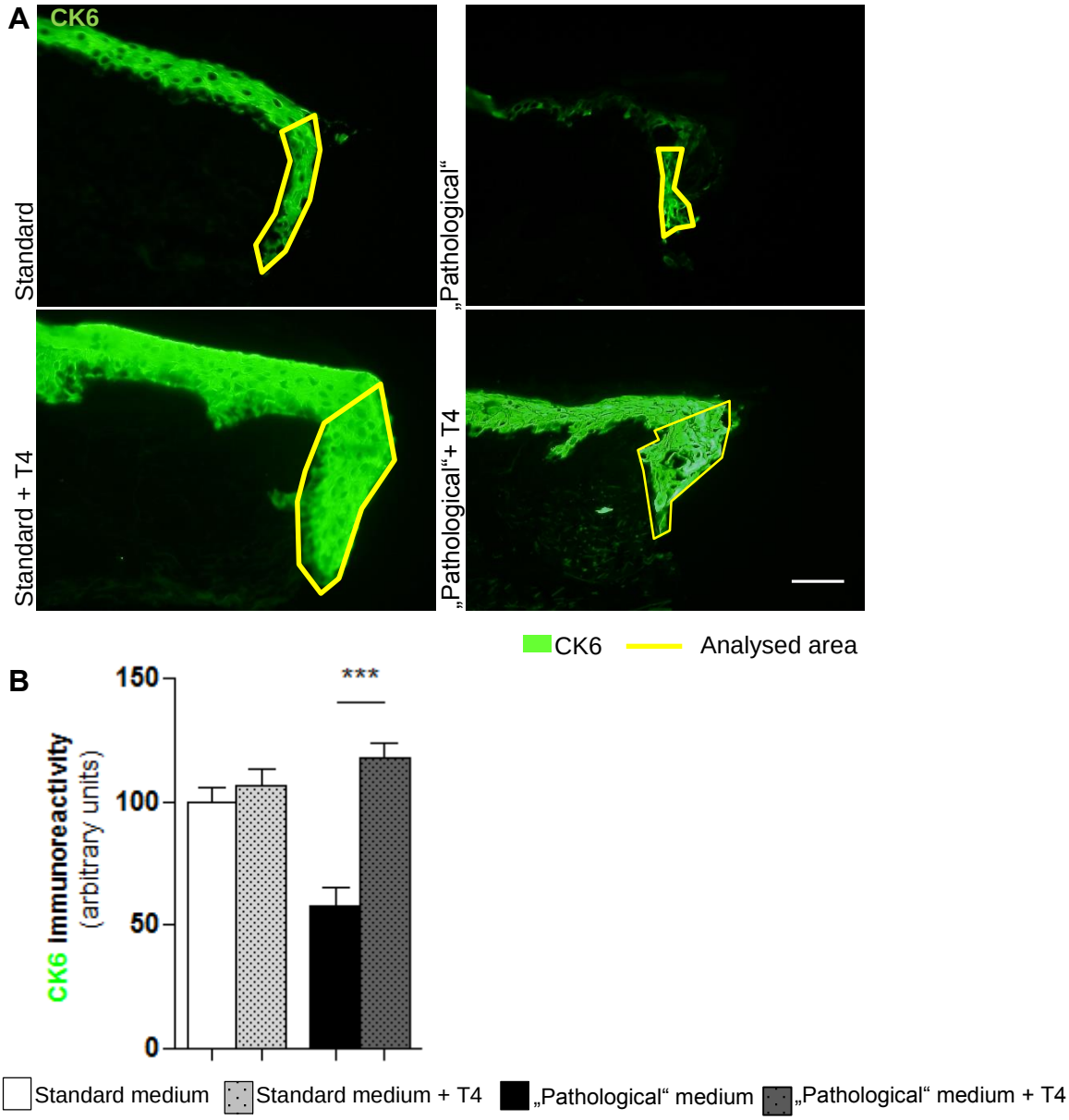

Figure 33 Cytokeratin 6 immunoreactivity was increased by thyroxine supplementation to the “pathological” medium

(**A**) Immunofluorescent cytokeratin 6 (CK6) staining of skin sections showing right wound edges and epithelial tongues (ETs). Green fluorescent immunoreactivity (IR) represents the CK6 positive signal and appears brighter in the skin from thyroxine (T4) supplemented medium conditions. The CK6 positive IR was assessed in the ETs (yellow continuous line) by quantitative immunofluorescence morphometry. (**B**) Statistical analyses demonstrate the assesment of the CK6 IR comparing standard versus (vs.) standard + T4 conditions and “pathological” vs. “pathological” + T4 conditions. All results were normalised to the standard medium (=100%). CK6 protein expression trended to be higher under T4 supplemented medium conditions. Upregulation of CK6 IR was significant under “pathological” conditions.

Scale bar: 50 µm; *** p≤0.001; pooled data from 3 different patients, 6 punches, 34-35 skin sections; mean ± SEM; p-value was calculated by Mann-Whitney-U test for unpaired samples.

5.1.8 Angiogenesis can be enhanced by thyroxine in particular under "pathological" conditions

As it was described before (Kassem et al., 2012; Liu et al., 2014; Safer, 2013), TH can initialize angiogenesis. After we evaluated that angiogenesis was severely impaired under "pathological" medium conditions, evaluations were performed in the same manner by CD31/DAPI staining, which was quantified by the three categories: (i) mean intensity of CD31 IR, (ii) total number of CD31 positive nuclei and (iii) total number of lumina.

The effects of T4 on angiogenesis can be confirmed in the category of CD31 IR. The dermal reference area of the T4 supplemented medium showed a significantly higher IR when medium was supplemented by T4 (**Figure 35**).

The total number of CD31 positive endothelial cells was significantly higher in the T4 supplemented "pathological" medium and showed a same trend in skin from standard medium conditions but was not significant (**Figure 36**). The criteria of the amount of lumina indicated also to be higher under the influence of T4 but were not significantly different (**Figure 37**).

These findings provide that T4 can significantly increase angiogenesis under "pathological" medium conditions.

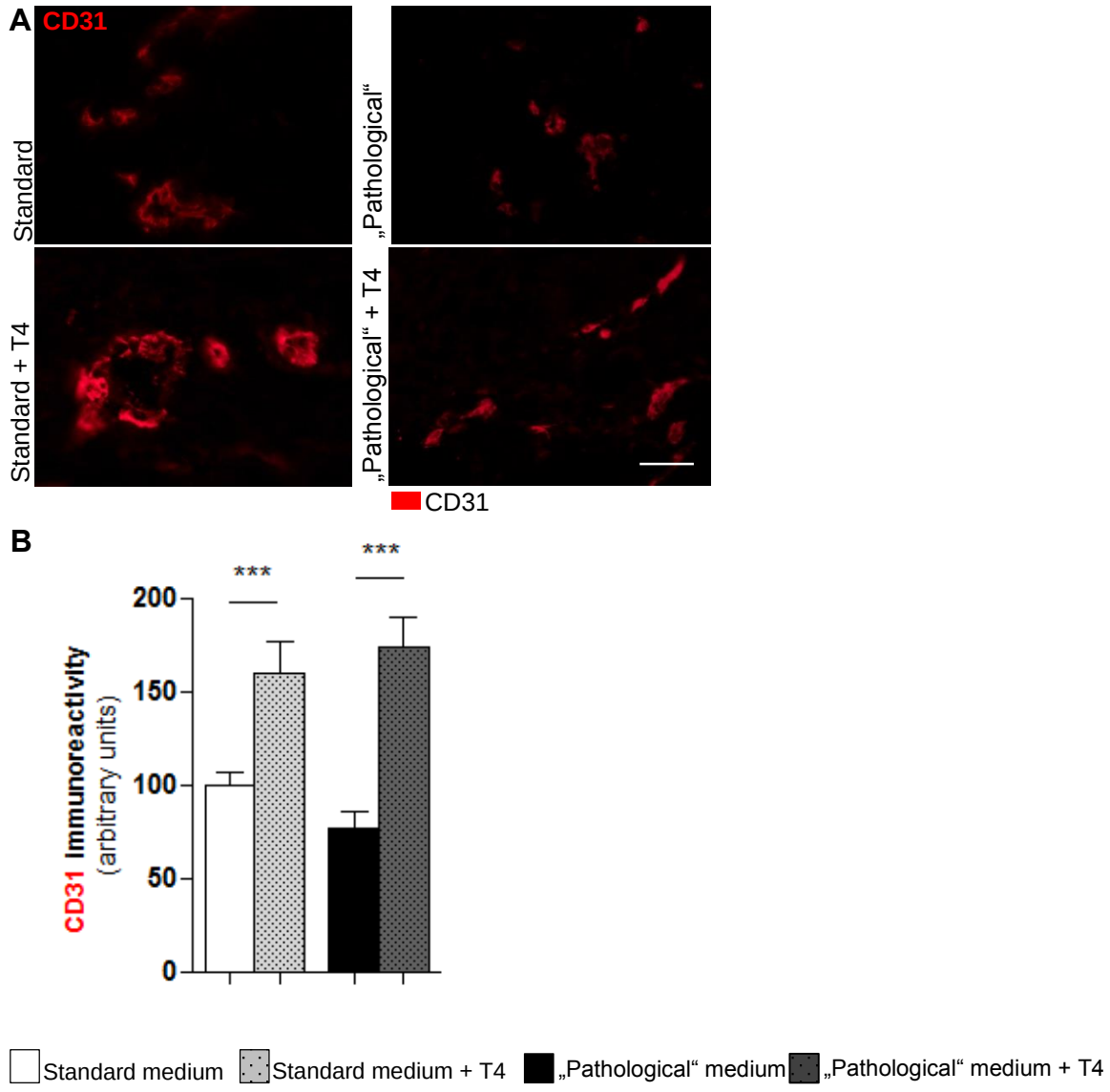

Figure 34 CD31 immunoreactivity increases under thyroxine supplementation to the medium

(**A**) Immunofluorescent CD31 staining of an analysed part of the dermis demonstrating a brighter red fluorescent CD31 positive signal in the skin of thyroxine (T4) supplemented medium conditions.

(**B**) Comparing the immunoreactivity (IR) of standard versus (vs.) standard + T4 conditions and “pathological” vs. “pathological” + T4 conditions normalised to the mean value of standard conditions to 100%. The T4 supplemented groups show a significant upregulation of the CD31+ IR which is even more obvious in “pathological” conditions.

Scale bar: 50 µm; ***p≤0.001; pooled data from 3 different patients, 39-54 skin sections; mean ± SEM; Mann-Whitney-U test for unpaired samples.

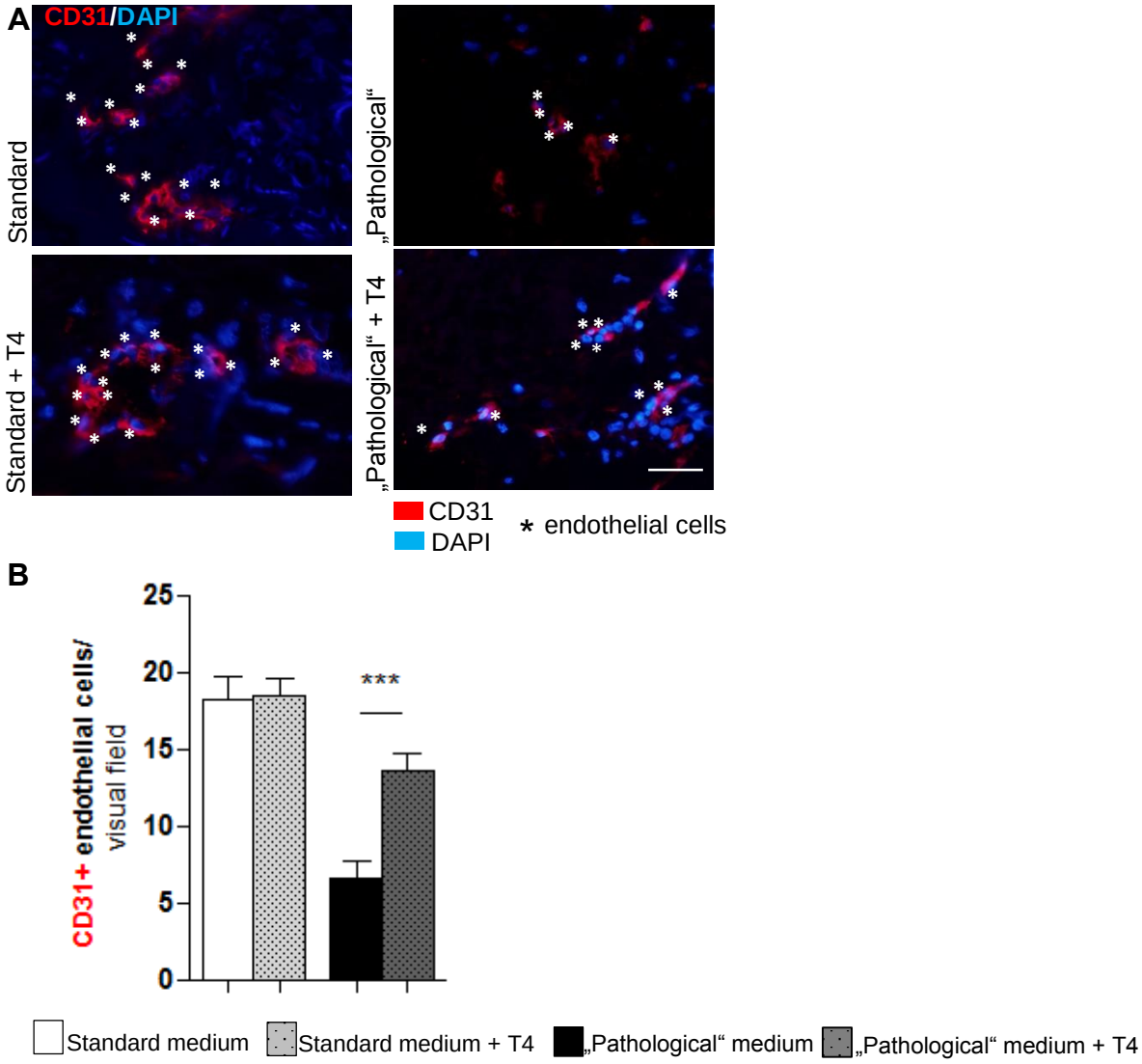

Figure 35 CD31 positive endothelial cells are more frequently detectable when “pathological” medium conditions were supplemented by thyroxine

(**A**) Immunofluorescent CD31/DAPI staining of a defined dermal area. An endothelial cell was counted for each blue fluorescent DAPI positive nucleus associated to a red fluorescent CD31 positive signal (white stars).

(**B**) The number of endothelial cells was assessed per visual field of three defined dermal reference areas comparing the results of standard versus (vs.) standard + thyroxine (T4) conditions and “pathological” vs. “pathological” + T4 conditions. The number of CD31 positive endothelial cells was not significantly different comparing standard and standard + T4 but in the T4 supplemented “pathological” group, significantly higher than in the “pathological” untreated group.

Scale bar: 50 µm; ***p≤0.001; pooled data from 3 different patients, 39-54 skin sections; mean ± SEM; Mann-Whitney-U test for unpaired samples.

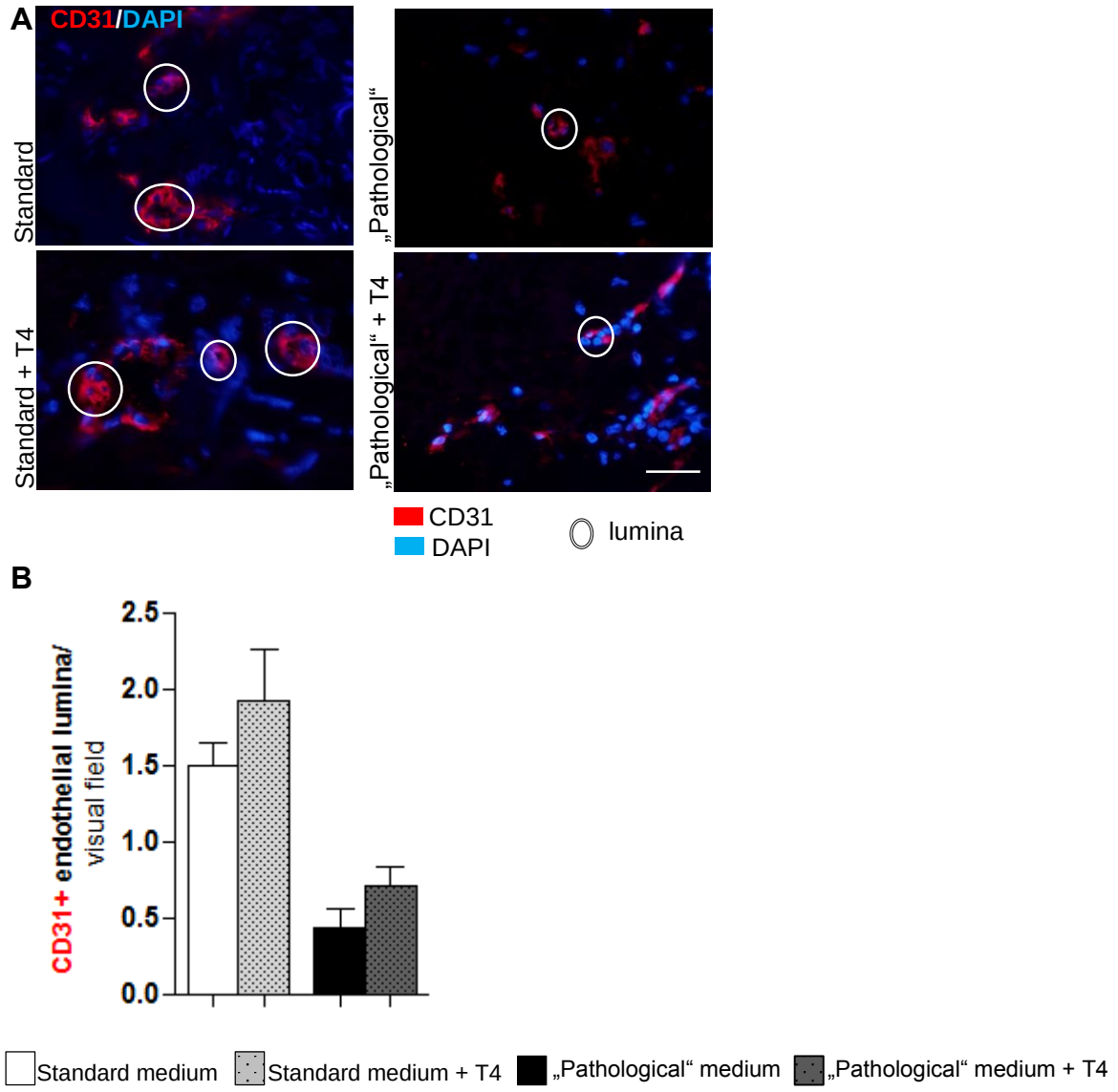

Figure 36 CD31 positive endothelial lumina were not significantly influenced by the addition of thyroxine to the medium

(**A**) Immunofluorescent CD31/DAPI staining of a defined dermal area. An endothelial lumen (white encircled) was counted for blue fluorescent DAPI positive nuclei associated to a red fluorescent CD31 positive signal forming a lumen.

(**B**) The number of lumina was assessed per visual field of three defined dermal reference areas comparing the results of standard versus (vs.) standard + thyroxine (T4) conditions and “pathological” vs. “pathological” + T4 conditions. There was no significant difference of CD31 positive lumina, neither in the standard vs. standard + T4 nor in the “pathological” vs. “pathological” + T4 even though the T4 supplemented groups trended to contain a higher amount of lumina.

Scale bar: 50 µm; ***p<0.001; pooled data from 3 different patients, 39-54 skin sections; mean ± SEM; Mann-Whitney-U test for unpaired samples.

6 Discussion

6.1 Modes of action of the “pathological” factors on wound healing in the applied *ex vivo* organ culture model and its clinical relevance

Hyperglycaemia, withdrawal of insulin, hypoxia and exposure to H_2O_2 all led to severe damage of the skin. The physiological WH process of human skin is a complex, multi-dynamic cycle. It is well-known that diseases like diabetes or angiopathy, and the resulting metabolic problems lead to WH disorders. The results of our “pathological” WH model were therefore not very surprising and showed serious defects of tissue integrity. To further outline the underlying processes of how tissue destruction is caused, the effect of each single “pathological” factor on cutaneous WH processes is going to be discussed in the following paragraphs.

6.1.1 Hyperglycaemia and the withdrawal of insulin impacts on wound healing

Tissue toxicity via hyperglycaemia leads to WH disturbances due to various mechanisms. One of these mechanisms is caused by the severe impairment of fibroblasts, which have critical functions in the later WH stages for ECM component production, secretion of GFs and for inducing angiogenesis. The recreation of “clinical hyperglycaemia” *in vitro* has been shown before, inhibiting fibroblast proliferation because of a resistance to respond to GFs like IGF and EGF (Berlanga-Acosta et al., 2013). In a cell culture model of fibroblasts under hyperglycaemic medium conditions, it was discovered that fibroblast proliferation, viability, collagen secretion and migration was highly decreased. This was interpreted as the consequence of the high expression of MMP9 via the exposure to hyperglycaemia. MMPs are proteinases which have a reducing function in the dynamic modification of the ECM (Xue and Jackson, 2015). Secretion of GFs such as VEGF was reduced in diabetic mice and also IGF-1 was considered to be reduced in the wound milieu of diabetic pigs with impaired WH (Velander et al., 2008). Furthermore, the loss of GFs like TGF-β, IGF-1 and PDGF leads to a disturbed collagen expression and thereafter to a defective ECM production (Berlanga-Acosta et al., 2013).

Another effect of hyperglycaemia among KCs leads to hyperosmolarity and consequently induces morphological changes like an enlargement of these cells and a

loss of orientation towards each other (Spravchikov et al., 2001). In our experiments, we also observed these changes in cell size and integrity. KCs appeared like “ballooning cells”, visible in the histochemical staining (PAS and H&E) of the “pathological” cultured groups. It was also described before that excessive glucose levels change nutrient transport, proliferation and growth where the latter two definitely correlating to our findings (Madonna et al., 2014).

Another tissue destructive effect of a glucose over-supply is the formation of advanced glycation end-products (AGEs). The formation of AGEs is also promoted by oxidative stress and leads to tissue dysfunctions by cross-linking of collagens, activation of macrophages and covalent bonding of lipoproteins (Siegenthaler and Amann-Vesti, 2006). Under “standard-diabetic” conditions of a latent hyperglycaemia, the formation of AGEs takes several weeks (Aronson and Rayfield, 2002) so that it could be criticised that this is not relevant for our experiments but there are investigations which considered that an over-supply of substrates can accelerate this process to a duration of only a few hours (Schiekofer et al., 2003). Accumulation of AGEs is one of the main pathomechanisms causing tissue damage in diabetic patients. Consequently, proteins of the ECM become invalidated, for example a decreased production of LN 332 which is a haptotactic substrate for KC motility located in the BL and could be associated with the impaired migratory potential of KCs of chronic wounds (Usui et al., 2008).

Additionally, matrix glycosylation was identified as an underlying mechanism of an increased cellular apoptosis, inhibition of cell proliferation, migration and angiogenesis in the skin from diabetic patients (Demidova-Rice et al., 2012a).

Insulin regulates blood sugar metabolism by the uptake of glucose into cells via transport glucose transport (GLUT) proteins (in case of KCs, GLUT 1, 2, 3 and 5) (Thorens, 1993) and also has WH promoting functions. Already at the beginning of the 20th century it was reported that WH can be enhanced by insulin and more recently that it stimulates human KC migration of *in vivo* incisional wounds on the back of mice (Liu et al., 2009). Additionally, insulin has pro-angiogenic functions via (nitric oxide) NO mediated regulation of angiogenic GF such as VEGF, FGF and TGF-β (Escudero et al., 2017; Lim et al., 2015). Summarising the effects of glucose and insulin on WH, it can be assumed that hyperglycaemia and loss of insulin can impact WH processes, which was confirmed by the results of the “pathological” cultured skin.

6.1.2 How hypoxia and the accumulation of reactive oxygen species interfere with wound healing processes

Given that tissue hypoxia, for example in peripheral vascular disease, can cause WH disorders, we simulated hypoxia in our cultures. Constantly low oxygen levels of 5% over the whole culture duration of 72 hours contributed to the altered skin constitutions under “pathological” conditions. The mechanisms of the way how hypoxia damages cutaneous cells and skin adnexa have been described before. A sufficient oxygen supply is indispensable for key WH processes like cell proliferation, bacterial defence, angiogenesis and collagen synthesis (Schreml et al., 2010). The essential role of O_2 in WH takes place in mitochondrial metabolism. Oxygen serves as the last electron in the glucose metabolism to produce adenosine triphosphate (ATP) which is needed for almost all cellular processes in the aerobic metabolism (Erecińska and Wilson, 1982). Morphological changes like hydrophic swelling and enucleation occur as well as the loss of E-cadherin proteins resulting in an extension of the intercellular spaces leading to cellular dysfunctions (Straseski et al., 2009).

H_2O_2 was added to the “pathological” medium periodically to induce the accumulation of reactive oxygen species (ROS) which respectively are a peroxide anion (HO_2^-), hydroxyl ion (OH^-) and a superoxide anion (O_2^-). In a balanced metabolic state, ROS is required for oxidative bacterial killing, and it is also involved in regulative processes during WH, like release of cytokines, cell proliferation and angiogenesis (Schreml et al., 2010). However, increased levels of H_2O_2 are known to reduce the migration and proliferation of fibroblasts which consequently impair complete wound closure (Thomas et al., 2009). Moreover, an excessive supply of ROS causes structural damage of the ECM and cell membranes and leads to early cell disintegration (Demidova-Rice et al., 2012b). Furthermore, ROS and proinflammatory cytokines induce the formation of MMPs and serin-proteinases, which again leads to ECM damage and also to an inactivation of GFs (Eming et al., 2007).

Furthermore, there is also evidence that insulin resistance and oxidative stress through H_2O_2 are likely to result in endothelial damage and cellular dysfunction (Lim et al., 2015). We could also prove this in our assay with high significance.

6.1.3 A summary of the “pathological” tissue effects on the wounded skin of the *ex vivo* organ culture model

Regarding the fact that the skin punches of our “pathological” groups were exposed to all of the above described WH impairing factors at once, signs of tissue destruction are severe. The “pathological” WH model is characterised by a high rate of cell death. The high number of apoptotic cells in the epidermis fits to the increased amount of dyskeratotic KCs and split formation. By contrast, proliferative cells under “pathological” conditions are a rarity. In our studies, the high number of apoptotic cells is therefore an explanation for the very small developed ETs. The fact that CK6 is hardly expressed in the “pathological” cultured skin also fits to the results described above and probably due to that there are also less functioning cells which are still capable to produce CK6. Angiogenesis is the essential part of the WH process. Endothelial cells are also altered by the cell death and consequently there are only a few blood vessel cross-sections in the dermis of skin cultured under “pathological” culture conditions which are formed of endothelial cells.

These results of our “pathological” WH model mirror how hyperglycaemia, loss of insulin, hypoxia and ROS-enriched tissue disturb the regular cell metabolism and hinder the regeneration of wounded skin. The investigations which were performed for the experiments of this thesis contribute to the fact that the applied “pathological” factors lead to severe WH interruptions.

6.2 Mechanisms of action of thyroxine on wound healing in the applied *ex vivo* organ culture model and its clinical relevance

With the aim of investigating a candidate WH promoter, we chose T4 for a pilot test study with the “pathological” WH model. Reepithelialisation, evaluated by length and area of the ET was significantly increased in the skin tissue of T4 supplemented medium under standard and “pathological” conditions. This leads to the suggestion that T4 promotes KC migration and thereby wound closure. In the experiments during the medical dissertation of Zhang, GY (2013) it was also shown that medium supplementation of T4 enhances the ET formation in terms of length and area of wounded human skin and an increased IR of cortactin was verified. Migrating KCs express this actin-binding protein, cortactin in their lamellopodia and it serves as a substrate for the activating protein Src-kinase (Ceccarelli et al., 2007; Gendronneau et

al., 2008). The effect of T4 on migratory processes in other cell types has already been investigated before. The migration of neuronal cells has been shown to be regulated by T4 by influencing the conversion of soluble actin to F-actin which is necessary for cell migration (Farwell et al., 2006). There are more investigations which confirm that T4 can stimulate the migration of various cell types (Bohnsack and Kahana, 2013; Liao et al., 2010) and in consequence are in line with our results as well as animal models which also describe a positive effect of THs on wound closure. First, T4 has been shown to have a positive effect compared to untreated groups in mice and rats (Erdoğan et al., 1999; Safer et al., 2005). Second, T3 enhanced WH processes in guinea pigs most likely through wound contraction (Kassem et al., 2012).

Proliferative cells were recognized to be increased by medium supplementation of T4 under “pathological” conditions. Under standard conditions, however there was a trend of a higher rate but without any significance. Significant increase of proliferative cells under “pathological” but not under standard conditions could be due to several reasons. One explanation is that the number of proliferative cells is far too low in “pathological” conditions so that the difference of a slightly increased number would become visible much more easier. Another reason is a potentially high variance between the values and a relatively small number of patients (namely three). Evaluation of apoptosis shows a reciprocally proportional outcome with a high significance in the “pathological” medium groups. This could be due to the very high values which were analysed in the skin from “pathological” medium conditions leading to a higher contrast between the apoptotic cell values. The promoting effect of T4 on proliferation of human KCs in an *ex vivo* organ culture has already been proven before (med. diss., Zhang, 2013). Here, the results were significant under standard conditions which underlines that the trend of our results of T4 having a positive effect on proliferation seems to be correct. Also the proliferation of KCs of the human HF matrix are impacted by THs. The proliferation of many more different cell types was reported to be influenced by THs *in vitro*, for example fibroblasts (Safer, 2013), cardiac fibroblasts (Yao and Eghbali, 1992), thyroid cells (Lin et al., 2007) and breast cancer cells (Verga Falzacappa et al., 2009). The mechanism behind this proliferative effect of T4 has been investigated and explained by plasma membrane-initiated actions via the receptor on integrin αvβ3 (Cheng et al., 2010).

Our findings of a decreased number of apoptotic cells are in line with van Beek et. al (2009) who found a reduced rate of KCs in the HF matrix under the influence of 100 nM T4. The role of T4 to affect apoptosis is also highly present in studies dealing with the development of neuronal tissue. A hypothyroid status during embryonic development causes deficiencies in the capability of the brain (Ahmed, 2015). By an investigation of the cell cycle of early differentiated granule neurons it was found that T3 prevented apoptosis by the TH promoted expression of Bcl-2, a protein which is responsible for preventing cell-death (Muller et al., 1995).

Knowing that there is a TH response element (TRE) in the keratin gene of epithelial cells that regulates CK expression it can be assumed that an alteration of the TH supply in the medium of a culture system affects CK6 expression. According to this fact, there is already some evidence that CK6 IR is increased in wounded human skin epithelium by T4 (med. diss., Zhang, 2013). T4 and T3 also have been shown to have the same enhancing effect on the CK6 expression in the human HF epithelium. Furthermore, the effect of an upregulated CK6 expression was also observed after the topical application of the hormone on wounds of mice (Safer et al., 2005). Our findings confirm the previous studies, in particular the results of the “pathological” cultured skin punches being highly significant. Under standard conditions, treated by T4 supplementation, there is also an upregulated tendency of CK6 IR compared to those cultured without T4 but this is not significant. This is the same phenomenon as the results of the Ki-67 positive, proliferative cells where the “pathological” medium groups have shown clearly significantly higher results in comparsion to the T4 treated groups of standard medium conditions.

Finally, a pro-angiogenic effect of T4 can be assumed. In all three assessed categories which were applied to analyse angiogenesis, T4 seemed to have an enhancing trend even more under “pathological” medium conditions. The results were significant in terms of an upregulated IR by the influence of T4 and an increased number of endothelial cells under T4 supplemented medium conditions was significant under “pathological” conditions. Even if the results of endothelial cells and lumina were not significant under standard medium conditions, there is evidence that T4 has a promoting effect on angiogenesis. Underlying mechanisms can be explained by elucidating the function of HIF-1α which becomes upregulated in response to tissue hypoxia. This was induced in our “pathological” medium and interestingly the

transcriptional factor (HIF-1α) also serves as an important stimulus for angiogenesis in WH processes (Andrikopoulou et al., 2011). HIF-1α is expressed in the human epidermis (Rezvani et al., 2011) and there also is a connection between T4 and HIF-1α which can be stimulated by the TH via activation of the phosphatidylinositol 3-kinase (Moeller et al., 2006). Another cellular mechanism which is responsible for the pro-angiogenic effect of T4 is the cell surface receptor integrin αvβ3, which mediates the promoting effects of THs on endothelial cells and vascular smooth cells. Stimulation of this receptor on endothelial cells stimulates intracellular transcription of pro-angiogenic GFs, like bFGF and VEGF (Cheng et al., 2010). Referring to the criteria of evaluating the number of lumina, it has to be considered that the formation of tubular channels and connection to each other by forming a vessel loop usually begins around the third day post-wounding (Reinke and Sorg, 2012). For this reason, a detection of lumina in our skin on 3-day culture duration might be too early.

However, the mechanisms of action and the exact signalling pathways of wounded human skin under “pathological” conditions remain to be further investigated in additional functional assays.

6.3 Classification of the “pathological” *ex vivo* wound healing model in the current state of the art

Next, we want to compare our newly developed “pathological” WH model to other pre-existing “pathological” WH models. The field of applied “pathological” WH models in general does not offer a huge variety. All investigated studies reveal many limitations in regard to clinical reality. Starting with the most rudimentary form of WH models, the scratch assays, there are models which apply human cells from diabetic donors or human KCs which are cultured under hyperglycaemia. The method of applying fibroblasts of patients with diabetic foot ulcers was utilised to investigate explicit gene expression patterns and disease specific phenotypes (Stephens, 2010) and by exposing KCs to hyperglycaemia it was proven that glucose impairs fibroblast and KC migration during WH in a dose dependent manner (Kruse et al., 2016). These scratch assays are less laborious, without the need of animals which is more time-, money- and space-consuming and offer the possibility of investigating isolated mechnisms of molecular processes. Still they only involve one kind of cell type and therefore lack the contribution of other neighbouring tissue structures to the WH process.

Looking for an organ culture model which is close to our model, a porcine model cultured under hyperglycaemic and hyperosmolar conditions has been established lately to investigate the effect of triterpene extract on WH (Ueck et al., 2017). Different from the scratch assay, there is the advantage of observing the WH process in the interplay of all involved neighbouring tissue structures (skin cells and skin appendages). The skin tissue structure and WH process of pigs is similar to those of humans but is obviously not the same. Additionally, it has to be mentioned that some systemic events contributing to WH, for example invading immune cells, the state of perfusion and immune cells, can only be demonstrated by *in vivo* WH models.

Exisiting *in vivo* WH models are mainly indicating the perspective of diabetes. The most common ones which are created by genetic programming are the Akita, NONcNZ010 and *db/db* mice. In other animal models, with rodents, pigs or rabbits, diabetes was caused by inducing cell death of pancreatic β-cells (Michaels et al., 2007). They have led to important findings about cellular processes of failed diabetic WH, including the role of macrophages and angiogenesis (Gallagher et al., 2007; Gordon, 2003). Nevertheless, those models do not reflect any other “pathological” factors than the diabetic issue and are not utilising human skin.

There is also an *in vivo* model of WH with human skin in genetically modified skin of humanised mice (Escámez et al., 2004). Here, the WH promoting ability of KGF was confirmed and the model was established as being well suited for studying WH but it has to be mentioned that this model does not include any “pathological” factor and even if it is *in vivo*, it does not include human skin.

Finally, tissue equivalents from engineered skin have to be taken into consideration. They have the advantage of a standardised high comparability and are easily reproducible without any ethical barriers, but one main disadvantage is that they do not incorporate skin appendages which play a significant role in WH (Li et al., 2017).

It is understood nowadays, that there are many more factors which contribute to chronic WH which are also missing in all of the “pathological” WH models. One of them is the aspect of ageing. There is evidence that age related falling oestrogen levels contribute to impaired healing in male and female patients (Emmerson and Hardman, 2012). Social isolation which is very often related to aged patients leads to lower cortisol levels which cause a decrease of KGF and VEGF, in turn, leading to WH disorders (Pyter et al., 2014). Another missing factor which is commonly missing in

current “pathological” WH models is the contribution of a changed bacterial microbiota. There is evidence that the altered biofilm of infected wounds on diabetic mice correlated with delayed WH (Grice 2010). Since the translation from rodents to humans has to be interpreted very carefully, more investigation has to be carried out to include human skin. A clinical trial about the microbiota of diabetic foot ulcers showed that an instability of microbiota community was associated with faster healing and better outcomes (Loesche et al., 2017).

It is not only the last case about the chronic wound microbiome, which provides evidence that we are still lacking comprehensive “pathological” WH models which mimic clinical reality as closely as possible, thereby leading to better therapeutic opportunities in treating the huge number of patients with chronic wounds.

6.4 Conclusion and future perspectives for wound healing studies

We have developed a pragmatic organ culture assay that quickly transforms viable biopsy-wounded human skin into a “pathological” state where WH is severely impaired. This novel pre-clinical test system, which is characterised by severe hypoxia, excessive ROS levels, hyperglycaemia and insulin withdrawal, can serve as a surrogate assay for probing the efficiency of candidate WH promoters under well-standardised and clinically relevant *in vitro* conditions that imitate major elements of diabetic, hypoxic, and ROS-damaged human skin. With this assay it should become possible to achieve a previously unattainable predictability during pre-clinical testing for estimating the chances of the test agent to actually promote WH even in chronic human skin ulcers. T4 as an already established WH promoting agent was applied successfully in this assay. It was proven that its positive healing effect is not only working in a physiological wound environment but also in massively metabolic disorder milieus. The efficiency of other WH accelerating substances like IL-22 (Avitabile et al., 2015), pro-insulin C-peptide (Lim et al., 2015), EPO (Hamed et al., 2014) can be tested with the “pathological” WH model. Furthermore, read-out parameters could be added depending on which part of the WH process it is aimed to investigate in more detail. For judging migratory processes this could be performed with an f-actin/cortactin stain which has been established as a reliable marker for cell movements (Gendronneau et al., 2008) and pro-angiogenic actions could be profoundly analysed by bFGF/VEGF and FGFR1 immunostainings to provide just two examples of multiple (immune-)

histochemical staining which could be applied to analyse more details about “pathological” WH in human skin. Since there are many more factors which affect WH, another future variation could be to investigate the WH disorders caused by radiation or excessive inflammation by exposing skin to x-rays or respectively to TNFα. Finally, we are able to assume that T4 has WH promoting effects but the long-term target is indeed to apply this substance in clinical medicine so that it can accelerate the healing time of a chronic ulcer. To reach this aim more studies of how, for example, T4 works systemically when it is locally applied and which vehicle would be optimal to apply T4 on a wound (patch, cream, foam or even a subcutaneous injection) have to be performed in subsequent investigations.

6.5 Critical remarks

Regarding the technique of tissue removal in our WH model, an improvement could be performed by replacing the punch by an ablative laser which cuts the skin fragments in a more precise and more standardised way. This was already performed with a non-sequential fractional, ultrapulsed CO_2 laser which cut out precisely equal skin equivalents for a 3D organ culture skin model (Marquardt et al., 2015). Another technical issue is that the skin punches floated at the air-liquid interface in the culture medium without being fixed, which facilitates the case that punches could have turned upside down, which possibly leads to altered cutaneous WH processes. This could have been improved by developing a retainer to hold the skin punch permanently and precisely at the air-liquid interface and it also warrants a complete surrounding of the tissue by the culture medium.

Regarding the medium conditions, measuring pH values or implementing lactat dehydrogenase (LDH) test before and during the culture duration, could have been interesting to document and thereby further analyse the quality of culture medium. Even if all skin fragments were put into the William’s E medium directly after surgery, kept at a temperature of 4°C and processed for culture preparation not longer than 24 hours after surgery, changes in pH value of the medium could have led to damages in skin tissue viability (med. diss., Zhang, 2013). This could have been monitored by an indicator paper in a convenient and technically easy way. During and after the organ culture, we also intended to check the medium conditions by measuring the amount of LDH concentration. An increased LDH inside the medium would indicate a higher rate

of cell death which would be assumable to take place in the “pathological” medium. This can be evaluated by different techniques for example by LDH cytotoxity colorimetric assay or by LDH cytotoxity fluorometric assay.

The advantages of using human skin instead of murine or skin equivalents have already been outlined but the number of available human skin tissue is limited and led us to a delay of our experiments while waiting for new tissue to arrive and made us carefully evaluate which further investigation we are able to perform next. Another critical point about using human skin, is an ethical issue which of course has to be strictly kept but nevertheless scientists are deprived to get to know crucial information about the health status of the patients. Besides name, sex, age and the place of where the surgery took place, we do not know if there might have existed underlying diseases like diabetes or thyroid disorders. But since the results of all three patients were in line and pooled data was without any high variances, we assume that there was not any underlying disease among our investigated patients.

Asking the question whether these circumstances we created are really comparable to the WH milieu of chronic ulcers, we have to admit that there are inevitable differences. Regarding tissue damages of diabetic patients, these are long-term damages which are difficult to recreate in a time period of 72 hours. With the excessively high concentrations of the “pathological” factors (for example glucose concentrations exceeded the physiological level by more than 10 times), we tried to conquer this barrier to accelerate the “pathological” processes.

It is also remarkable that we chose to investigate one concentration of our testing agent T4 and that this concentration is in the physiological range of normal T4 serum levels (Avlos et al., 1986), which in turn leads to the question whether the medium conditions of the not T4 supplemented groups were in a hypothyroid state. However, our experiments had the first intent to establish a “pathological” WH model and the application of T4 was performed to prove its practicability. We relied on previous experiments in which different concentrations of T4 (10 nM, 100 nM and 1000 nM) were tested and the application of 100 nM showed the most significant results (med. diss., Zhang, 2013). Even though more concentrations and different ways to apply T4 to wounds need to be tested, this thesis provides several reasons to assume that T4 has a positive effect on “pathological” WH of human skin *ex vivo*.

7 Summary

The treatment of impaired cutaneous wounds, particularly in the context of chronic venous ulceration and an aging population, represents a major medical challenge. Despite this, satisfactory human models of cutaneous wound healing are lacking. Moreover, current models overlook the hypoxia, hyperglycaemia, poor perfusion and oxidative stress which together all contribute to poor wound healing. Therefore, we decided to develop an *ex vivo* “pathological” human skin model in which to examine wound healing and identify wound healing promoting agents.

The thesis presents the evaluation of the development of a pragmatic surrogate wound healing model. Organ cultures were run with human full-thickness skin punches under the influence of hypoxia, hyperglycaemia, withdrawal of insulin and oxidative stress for three days. The “pathological” changes were evaluated according to the following criteria: dyskeratosis, split formation, length and area of the epithelial tongue, proliferation/apoptosis, formation of the early wound healing related protein 6 and angiogenesis.

After we had established this model, with the matching composition and concentration of impact factors, a potential wound healing promoter was tested. Supplementing T4 to the standard and “pathological” medium resulted in improved wound healing, defined by the criteria of length and area of the epithelial tongue, proliferation/apoptosis, expression of the early wound healing related protein keratin 6 and angiogenesis.

Also presented in the context of this thesis are the fundamental current scientific knowledge about the organic function of the skin and the present understanding of chronic wounds and wound healing.

8 Zusammenfassung

Die Behandlung von kutanen Wundheilungsstörungen, insbesondere im Zusammenhang mit Begleiterkrankungen, wie Diabetes mellitus und chronisch venöser Insuffizienz bei einer generell alternden Bevölkerung, stellen eine große medizinische Herausforderung dar. Trotz dieser Tatsache gibt es zu wenig zufriedenstellende Wundheilungsmodelle mit menschlicher Haut. Weiterhin berücksichtigen gängige Wundheilungsmodelle Faktoren wie Hypoxie, Hyperglykämie, eine unzureichende Durchblutung und oxidativen Stress nicht, obwohl diese meist ursächlich für die Chronizität einer Wunde sind. Aus diesem Grund kam es zu der Entscheidung ein „pathologisches“ *ex vivo* Modell mit menschlicher Haut zu entwickeln, womit im weiteren Verlauf die Wundheilung an sich und Substanzen auf ihre wundheilungsfördernde Wirkung an chronischen Wunden getestet werden können.

Die vorliegende Arbeit präsentiert die Entwicklung und die Evaluation eines Wundheilungsmodells, das als Organkultur mit Stanzbiopsien von humaner Vollhaut, 3 Tage dem Einfluss von Hypoxie, Hyperglykämie, dem Entzug von Insulin und oxidativem Stress ausgesetzt wurde. Die unter „pathologischen“ Bedingungen kultivierten Hautproben wurden im Hinblick auf Dyskeratosen, subepidermale Spaltbildung, Längen- und Flächenwachstum der epithelialen Zunge, Proliferation/Apoptose, Ausprägung des in der frühen Phase der Wundheilung ausgeprägten Proteins Zytokeratin 6 und Angiogenese beurteilt.

Nachdem dieses Modell mit den passenden Zusammensetzungen und Konzentrationen von Einflussfaktoren etabliert wurde, kam es zur Anwendung, indem eine potenziell wundheilungsfördernde Substanz, das Schilddrüsenhormon Thyroxin, getestet wurde. Das Hinzufügen von Thyroxin zum normalen und „pathologischen“ Medium führte in den Hautproben teilweise zu einer verbesserten Wundheilung, was durch die Beurteilung von Längen- und Flächenwachstum der epithelialen Zunge, Proliferation/Apoptose, Ausprägung des akuten wundheilungsassoziierten Proteins Zytokeratin 6 und Angiogenese festgestellt wurde.

In diesem Zusammenhang wurden in der vorliegenden Arbeit auch der aktuelle Stand der Wissenschaft über die kutane Wundheilung, sowie chronische Wunden und generelle Strukturen und Funktionen der Haut dargestellt.

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10 Annex

Name	Company
Aceton	Roth
Alcohol	Merck
Aqua dest.	Merck
Antibody diluent	Dako
Cryomatrix	Shandon
DAPI (4‘,6-diamidino-2-phenylindole)	Roche
Eosin E	Sigma
Eukitt	Sigma
Fluormount-G	Southern biotech
Glucose	Sigma
Goat normal serum	Dako
Hydrochloride acid	Merck
Mayer‘s Haemalaun	Merck
Mounting medium Faramount	Dako
Paraformaldehyde	Merck
Periodic acid	Merck
Schiffs reagent	Merck
Sodium chloride	Merck
Sodiumbisulfit	Merck
Xylol	Merck

Table 3 Chemicals

Name	Host	Dilution	Method	Source	Clone
Ki-67	Mouse	1:20	IF	Dako	MIB-1
Anti-Digoxigenin	(TUNEL-Kit)	59:56	IF	TUNEL-Kit	TUNEL-Kit
CD31	Mouse	1:30	Indirect IF	Dako	MO823
Keratin 6	Mouse	1:10	Indirect IF	PROGEN	Ks6.KA12

Table 4 Primary antibodies

IF: immunofluorescence; TUNEL: Tdt-mediated dUTP-biotin nick end labelling

Name	Conjugated with	Dilution	Company
Goat anti-mouse	FITC	1:200	Jackson ImmunoResearch
Goat anti-mouse	Rhodamine	1:200	Jackson ImmunoResearch

Table 5 Secondary antibodies

PBS (pH = 7.2) Phosphate buffered saline	8.0 g sodium chloride 1.8 g sodium dihydrogen phosphate monohydrate *Aqua dest.* ad 1000 ml
TBS (pH = 7.6) Tris buffered saline	6.1 g Trizma Base 8.8 g sodium chloride *Aqua dest* ad 1000ml

Table 6 Buffers

Name	Company
Hydrocortisone	Sigma
Insulin	Sigma
L-glutamine	Gibco™ (Invitrogen) Corporation
Penicillin G/streptomycin	Gibco™ (Invitrogen) Corporation
Triiodothyronine	Sigma
Thyroxine	Sigma
William´s E Medium (2,2 g/l NaHCO3)	Biochrom KG

Table 7 Organ culture reagents

Name	Company
Bright field microscope	Krüss
Cell culture incubator Autoflow	Nuaire™
Cell culture incubator Whitley A35	Don Whitley scientific
Centrifuge 5810	Eppendorf
Cover glasses	Menzel-Gläser
Cryostat	Leika
Fluorescence microscope	Biozero-8000 Keyence
Deep freezer KTL	Kryotec/Kryosafe
Forceps	PFM
Invivo 2400 Hypoxie Workstation	Ruskinn Technology
Laboratory scale	Sartorius AG
Laminar airflow	ScanLaf
Magnetic stirrer	IKA®
Microscope slide	Menzel-Gläser
Petri dish	SARSTEDT
Pipettes (10 µl, 100 µl, 1000 µl)	Eppendorf
Pipette tips	SARSTEDT
Punch (4mm)	PFM
Reaction vessels	Eppendorf
Refrigerator-freezer	Simens AG
Safety cabinet Clean Air	Thermo
Scalpel blade	Martin
Scissors	PFM
Six-well-plate	Nunc
Vortexer IK	A®MS3 basic

Table 8 Equipment

9 Danksagung

Mein ganz besonderer Dank gilt Frau Prof. Dr. med. vet. Jennifer Elisabeth Hundt (Lübecker Institut für Experimentelle Dermatologie). Ihre vielseitige Inspiration, ihr verantwortungsvolles Engagement und zahlreichen Ratschläge und Anregungen haben maßgeblich zur Erstellung dieser Dissertation beigetragen. Die Zusammenarbeit war stets und auch über das Fachliche hinaus eine große Bereicherung.

Herrn Prof. Dr. med. Ralf Paus (Dept. of Dermatology & Cutaneous Surgery, University of Miami Miller School of Medicine, USA and Centre for Dermatology Research, University of Manchester, UK) danke ich für die Vergabe des Promotionsthemas und für die wichtigen Ratschläge zur Durchführung und Auswertung der Experimente, sowie für die Hilfe bei dem Erstellen des wissenschaftlichen Artikels.

Herrn Prof. Dr. med. Detlef Zillikens, dem Direktor der Hautklinik (Klinik für Dermatologie, Allergologie und Venerologie, Universität zu Lübeck), gilt meine Dankbarkeit für die Möglichkeit, in seiner Klinik als Doktorandin arbeiten zu können.
Herrn Prof. Dr. med. Ralf Ludwig, dem wissenschaftlichen Leiter des Lübecker Instituts für experimentelle Dermatologie, gilt ebenfalls meine Dankbarkeit für die Möglichkeit, in seinem Institut als Doktorandin arbeiten zu können.

Herrn Prof. Dr. med. Dr. h. c. Thomas Luger, dem Direktor der Hautklinik des Universitätsklinikum Münster (Klinik für Hautkrankheiten - Allgemeine Dermatologie und Venerologie, Universitätsklinikum Münster) danke ich für die Erlaubnis zur Mitbenutzung seiner Laboreinrichtungen, die mir dazu verholfen hat alle Auswertungen vollständig zu Ende zu führen.

Allen anonymen Spendern danke ich für deren großzügige Bereitstellung der Hautexzidate, die die Grundvorraussetzung zur Durchführung dieser Arbeit waren.
Bei den Mitarbeitern der ehemaligen AG Paus möchte ich mich bedanken, insbesondere bei Dr. rer. nat. Nancy Ernst, Antje Winter-Keil, Nadine Dörwald und

Claudia Kremling, die mir durch ihre Hilfe und technische Beratung die Möglichkeit zur Mitbenutzung diverser Geräte und Substanzen gegeben haben.

Dr. rer. nat. Jérémy Cherét und Dr. rer. nat. Marta Bertollini (Labor für Haarforschung und Regenerative Medizin, Universitätsklinikum Münster) danke ich für die Unterstützung zur Fertigstellung der Auswertungen der letzten Versuche in Münster.

Dr. med. Ewan Langan (Klinik für Dermatologie, Allergologie und Venerologie, Universität zu Lübeck) gilt mein ganz besonderer Dank für seine Anmerkungen und fachlichen Diskussionen, die mir zu einer kompetenten Auswertung und korrekten Darstellung der Experimente geholfen haben.

Dr. med. Guoyou Zhang (Department of Plastic and Reconstructive Dermatology, Shanghai, CN) danke ich für die Unterstützung bei der Planung der Versuche, besonders in der Anfangsphase.

Herrn PD Dr. med. Christian Rose (Klinik für Dermatologie, Allergologie und Venerologie, Universität zu Lübeck) danke ich für die besonders kompetente und geduldige Beratung bei der Beurteilung der histologischen Präparate, sowie für die Unterstützung bei der Literaturrecherche.

Bei Herrn PD Dr. rer. nat. Reinhard Depping (Institut für Physiologie, Universität zu Lübeck) möchte ich mich für die Bereitstellung des Hypoxie Inkubators bedanken, ohne den das “pathologische“ Wundheilungsmodell nicht möglich gewesen wäre und darüber hinaus danke ich für seine zuversichtliche und sympathische Unterstützung.

Meinen lieben Eltern Milena und Ulrich und meiner lieben Schwester Lara danke ich von ganzem Herzen dafür, dass sie mich während meines gesamten Studiums und meiner Promotion immer und überall unterstützt haben und mir in den entscheidenden Momenten das nötige Selbstvertrauen und die Motivation gegeben haben.

Meinem lieben Jan-Birger danke ich ganz besonders für seine endlose Geduld und dafür, dass er immer für mich da ist und an mich glaubt!

10 Curriculum vitae

PERSONAL DATA

Hannah Post
Born: April 23rd, 1988
in Gronau

CAREER

March 2016 – April 2018	Knappschaftskrankenhaus, Recklinghausen - Resident (“Assistenzärztin“) at the Department of Dermatology

EDUCATION - STUDIES

January 2016	Medical approbation
November 2014 – October 2015	Clinical internship (“Praktisches Jahr”) - Department of Surgery, • University Hospital Schleswig-Holstein, Campus Lübeck • St. Marien Hospital, Ahaus - Department of Internal Medicine, • “Kantonsspital” Luzern, Switzerland • Sana Clinic, Lübeck - Department of Dermatology • University Hospital Schleswig-Holstein, Campus Lübeck
May 2011-November 2015	Clinical part of medical studies at the University of Lübeck
03/2014 – 04/2014	Internship at the Department of Dermatology, University Hospital of Hamburg Eppendorf
03/2013 & 09 - 10/2014	Internship general medicine, Dr. Kuchar, Lohr am Main

08/2013 - 09/2013	Internship at the department of traumatic surgery, University Hospital Schleswig-Holstein, Campus Lübeck Internship at the Department of Paediatrics, Hospital Italiano, Buenos Aires, (Argentina)
04/2011	Physikum
09/2009	Beginning of medical studies at the University of Johannes Gutenberg University of Mainz

DISSERTATION

July 2012 – July 2013	Experimental thesis (experimental part), AG-Paus, at the Department of Dermatology, University of Lübeck & *Lübecker Institut für experimentelle Dermatologie* (Title: "Development and testing of a novel *ex vivo* assay for studying "pathological" wound healing in human skin") **Thesis advisor:** Prof. Dr. med. vet. Jennifer E. Hundt (Initially: Prof. Dr. med. Ralf Paus)

PUBLICATION

2012 & 2013	***6. & 7. Lübecker Doktorandentag***, University of Lübeck, (Poster presentation)
2013	**International Investigative Dermatology (IID) Meeting,** Edinburgh, UK, (Poster presentation)
2014	**5th symposium of regenerative medicine**, Lübeck, (Poster presentation)

EDUCATION - SCHOOL

06/2007	Abitur
1998 - 2007	Secondary School, Alexander-Hegius-Gymnasium, Ahaus
2004 - 2005	Forrest Hills Eastern High School, Grand Rapids, USA
1994 - 1998	Primary School St. Josef, Ahaus